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MESSAGE:

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Appellants:	Donald L. Wise, Debra J. Trantolo, David D. Hile, and Stephen A. Doherty		
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Appeal Brief; and
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Contents

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Cover: Scanning electron micrograph of PLGA-85:15 foam prepared by lyophilization of benzene solution, 10 KV. Figure 2 from the article by Hsu et al., pages 107–116, this issue.

Effect of polymer foam morphology and density on kinetics of *in vitro* controlled release of isoniazid from compressed foam matrices

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The purpose of this study was to compare the effect of polymer foam morphology and density prior to compaction on the kinetics of isoniazid (INH) release from the final high-density extruded matrices. The feasibility of preparing low density foams of several biopolymers, including poly(L-lactide) (PLLA), poly(glycolide) (PGA), poly(DL-lactide-*co*-glycolide) (PLGA), poly(γ-benzyl-L-glutamate) (PBLG), and poly(propylene fumarate) (PPF), via a lyophilization technique was investigated. Low-density foams of PLGA, PBLG, and a mixture of PLGA and PPF were successfully fabricated by lyophilization of the frozen polymer solutions either in glacial acetic acid or in benzene. The morphology of these foams depends on the polymer as well as the solvent used in the fabrication process. Thus, PLGA produces a capillary structure when lyophilized from benzene solution and a leaflet structure from glacial acetic acid, but PBLG yields a leaflet structure from benzene. Matrices were prepared by impregnating these foams with aqueous solutions of INH, removing the water by a second lyophilization, and then compressing the low-density INH containing foams by compaction and high-pressure extrusion. The resulting nonporous matrices had densities of approximately 1.30 g/cm^3. *In vitro* kinetics were in accord with the Roseman–Higuchi diffusion model and demonstrate that release rates depend on the initial foam density, while foam structure has little influence on the release kinetics. © 1997 John Wiley & Sons, Inc.

INTRODUCTION

The use of porous biopolymers as drug excipients in controlled-release systems, and as cell scaffolding in tissue engineering, has stimulated great interest in recent years.[1–3] The use of open polymeric foams in the preparation of release systems has been shown to minimize the initial burst and yield slower release rates. The reductions appear to be the result of effective trapping of the active agent within the matrix following high-pressure compaction of the drug-impregnated foam.[4] Porous biocompatible polymers also appear to serve as a scaffold for tissue regeneration and may function even more effectively by incorporating growth factors. One example, the healing of hard-tissue defects, was reported by Jamshidi et al.,[5] who used a porous coating of polylactide to control the release of coralline hydroxyapatite for the resurfacing of damaged articular cartilage. Several patents and articles regarding the use of porous PLGA as bone graft substitute devices have also been reported.[6–9]

To understand the factors which govern the release of active agents entrapped in compacted polymeric foams, we first explored several bipolymers by examining the feasibility of their foam formation, followed by foam characterization. These characterized foams were then systematically investigated by selecting several parameters which may affect the release of the active agent, including the selection of the biopolymers, the effect of solvent on the foam structure for dissolving polymers, and the effect of foam density on release rates. We also measured the diffusivities of the antituberculosis drug isoniazid (INH) in the resulting matrices by applying a kinetic model developed by Roseman and Higuchi.[10] We have shown this analysis to be applicable to release of the INH from cylindrical PLGA matrices.[4,11]

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MATERIALS AND METHODS

Materials

Isoniazid was purchased from Sigma Chemical Co. (St. Louis, MO). Poly(DL-lactide-*co*-glycolides), lactide to glycolide weight ratio of 85:15 (PLGA-85:15) and 75:25 (PLGA-75:25), were obtained from Polysciences (Warrington, PA). Poly(DL-lactide-*co*-glycolide) with a 58:42 (PLGA-58:42) weight ratio of lactide to glycolide was obtained from American Cyanamid Co. (Wayne, NJ). Poly(L-lactide) and poly(glycolide) were purchased from Medisorb (Du Pont Co., Wilmington, DE). Poly(γ-benzyl-L-glutamate) (PBLG) with a molecular weight of 150,000 was obtained from Sigma Chemical Co. Solvents (acetone, 2-propanol, and glacial acetic acid) were obtained from Fisher Scientific (Pittsburgh, PA). INH, PLLA, PGA, PBLG, and solvents were used without further purification. The PLGAs were purified via precipitation prior to use as described below. Poly(propylene fumarate) was synthesized by the direct esterification of fumaric acid (Fisher Scientific, Fair Lawn, NJ) with propylene glycol (1,2-propanediol; Aldrich Chemical, St. Louis, MO) by an adaptation of the procedure described by Sanderson.[12] Fumaric acid and propylene glycol were used as received.

PLGA purification

PLGA-85:15, PLGA-75:25, and PLGA-58:42 were purified prior to use. The polymer was dissolved in acetone (5 g/dL) and the solution slowly added to 2-propanol with continuous stirring. A volume ratio of 100 mL of polymer solution to 500 mL of 2-propanol was not exceeded. The fibrous precipitate was vacuum dried (<1 mm Hg) at room temperature for 48 h prior to use.

Low-density polymer foam preparation

Sufficient polymer was dissolved in a 100-mL lyoph-lock flask (Fisher Scientific) containing either 30 mL glacial acetic acid or benzene to form the desired concentrations. The solution was then frozen at −10°C, followed by lyophilization to remove the solvent. The conditions of lyophilization were as follows: the frozen polymer–solvent solution was kept in a ice bath under a vacuum of <1 mm Hg for at least 7 days. Solvent was collected in a solvent trap cooled in a dry ice–acetone bath at −78°C. After complete removal of the solvent as judged by the absence of the pungent odor, the polymer foam was removed from the flask for measurement of its bulk density, molecular weight (M_w), glass transition temperature (T_g), and morphology.

Foam characterization: density, molecular weight, glass transition temperature, and morphology

Foam density as a function of solution concentration was measured by liquid displacement. A sample of foam of weight W was immersed in a graduated cylinder containing a known volume (V_1) of water. Air was removed from the cylinder in a series of brief evacuation-repressurization cycles to force water into the interstitial spaces of the foam. Cycling was continued until no air bubbles were seen emerging from the foam. The volume difference then recorded ($V_2 - V_1$) represented the volume of polymer only. The water-impregnated foam was then removed from the cylinder and the volume (V_3) then recorded. The quantity $V_1 - V_3$, the volume of water held by the foam, was identified as the void volume, and $V_2 - V_3$ was the volume occupied by the polymer alone. Thus, the foam density, d_f, can be expressed as

$$d_f = \frac{W}{(V_1 - V_3) + (V_2 - V_1)} = \frac{W}{(V_2 - V_3)} \quad (1)$$

The densities of the solid nonporous polymers were determined by compacting samples at 49°C and 19,100 psi to form solid discs of known dimensions. The weights as well as the volumes of the discs were taken for computing the polymers' solid densities (d_p). The densities of PLGA-85:15, PBLG, and PLGA-85:15/PPF (50/50 wt %) were 1.80, 1.65, and 1.74 g/cm^3, respectively. Because ($V_2 - V_1$) represents the volume of solid polymer, the quantity W/d_p may be substituted in the above equation.

Molecular weight before and after foam formation was determined by gel permeation chromatography (GPC) on a Waters Associates Ultrastyragel 10^4 Angstrom column using a tetrahydrofuran mobile phase at a flow rate of 1 mL/min. Instrumentation, using a 600/600E system controller, consisted of a 410 differential refractometer, 468 tunable ultraviolet (UV)/VIS detector (the former was used for GPC), and dual pump multisolvent delivery system. Data analysis based on polystyrene standards for calibration was performed with the Maxima 825 software program.

Thermal analyses of the foams were conducted using a differential scanning calorimeter (DSC; Metler DSC/TG TA 4000) with a 5°C/min heating rate

Samples weighing about 5–10 mg were analyzed in aluminum pans each time.

Foam morphology was examined by scanning electron microscopy (SEM). Micrographs were taken on an Amray AMR-1000 scanning electron microscope. The interior structure of the foams was accessed by cooling in liquid nitrogen and fracturing them in the desired direction.

Impregnation of low-density polymer foams with INH

Isoniazid was incorporated into the low-density polymer foam by immersing the foam into an aqueous INH solution of known concentration. The solution was pulled into the pores of the polymer foam by repeated cycles of evacuation and repressurization. Water was then removed by a second lyophilization. Lyophilization was continued until the weight of impregnated foam remained unchanged. The gain in weight of polymer following impregnation was taken as the weight of INH incorporated into this low-density polymer.

The INH loading in this low-density polymer can be predicted by Equation (2), in which C is the INH aqueous concentration, ρ_P is the density of the solid polymer, ρ_F is the foam density, and F_v is the void fraction of the foam (densities and concentration in grams per milliliter). As can be seen, drug loading can be increased by increasing the void fraction (F_v) and also by increasing the drug concentration (C) in the impregnating solution.

$$\text{INH loading (w/w)} = \frac{C \cdot F_v}{\rho_F + C \cdot F_v} = \left[1 + \frac{\rho_F \rho_P}{C(\rho_P - \rho_F)}\right]^{-1} \quad (2)$$

Grinding and extrusion

The impregnated polymer foam was compressed by placing it between two smooth heated metal plates maintained at 1500 psi and 48°C by a heating tape for 24 h. The compression was accomplished by a hydraulic press equipped with a constant pressure controller (Compac Model MPC 40-1; Stenhoj Co., Denmark). The impregnated polymer foam after compaction was a flat, hard, white disk. The disk was dipped into liquid nitrogen for 2 min to make it brittle, and then ground while cold in a water-cooled Tekmar A-10 mill. The product was sieved through standard Tyler mesh screens to retain the 125–180-μm fraction.

The ground and sieved INH–polymer matrices were loaded into a cylindrical extrusion mold equipped with interchangeable dies. The assembled mold was then mounted onto the hydraulic press. The pressure was then set and the mold was heated by external heating tapes to the minimum temperature at which extrusion would occur at that pressure. The diameter of the extruded rods was approximately 2 mm.

In vitro procedure

One-centimeter lengths of the extruded rod were immersed in 40 mL of pH 7 phosphate-buffered saline (PBS) contained in a 50-mL plastic vial (Nalgene 3139-0050) (PBS composition: Na_2HPO_4, 0.0134*M*; $NaH_2PO_4 \cdot H_2O$, 0.0726*M*; NaCl, 0.0753*M* adjusted to pH 7.0 with NaOH or HCl). Vials were held in a thermostatted shaking bath (Shaking Water Bath 25; Precision Scientific) set at 37°C and operating at 40 cycles/min. Aliquot samples of 5 mL were removed at various intervals for spectrophotometric analysis of the INH content and replaced with an equal volume of fresh buffer. Each matrix was run in triplicate. Absorbance was measured at 262 nm, the observed maximum of INH in PBS, on a Cary 1 spectrophotometer (Varian). The extinction coefficient was previously determined by linear regression of a Beer–Lambert plot. In the concentration range of 15–80 μg/mL the extinction coefficient (slope) was 32.11 absorbance units (AU) per unit concentration (mg/mL), or 4404 L mol^{-1} cm^{-1}. The intercept was 5.97×10^{-3} and the correlation coefficient was 0.9997.

Measured absorption was converted to fraction released, *F*, by the following equation, which takes into account the quantity of INH discarded with each aliquot sample:

$$F_n = (\varepsilon M)^{-1} \left[A_n V + \sum_{i=1}^{n-1} A_i v \right] \quad (3)$$

where

M = initial weight of INH in the rod, mg

e = extinction coefficient, AU/mg ml^{-1}

A_n = absorbance of the nth aliquot sample

V = total volume of solution = 40 ml

v = volume of aliquot sample = 5 ml

sub i n indicates samples taken at times i . . . . . n.

Solubilities of INH in PLGA-85:15, PBLG, and PLGA–PPF

Isoniazid solubility in PLGA-90:10 was measured previously[11] to be 15 mg/cm^3 via a film-casting method. The same method was used again to determine the INH solubilities in PLGA-85:15, PBLG, and 50:50 wt % PLGA–PPF. Briefly, INH and the polymer were codissolved in 50 mL of methylene chloride. The solution was cast on a Teflon sheet to form a film. The air-dried film was then examined microscopically for the emergence of precipitated drug particles. INH particles were observed at 1.5 wt % in PLGA-85:15 films, 1.0 wt % in PBLG films, and 1.5 wt % in PLGA–PPF films, yielding 22.5 mg/cm^3 INH solubility in PLGA-85:15, 12.4 mg/cm^3 in PBLG, and 21.8 mg/cm^3 in PLGA–PPF.

RESULTS AND DISCUSSION

Five polymers were selected for their solubilities in glacial acetic acid and benzene: PLLA, PGA, PPF, PBLG, and several lactide-glycolide copolymers (PLGAs). Because of their superior biocompatibility and biodegradation, PLLA, PGA, and their copolymer, PLGA, are among the family of Food and Drug Administration–approved polymers that are used in resorbable sutures and drug delivery systems.[13] The use of porous PLGA-85:15 foam for preparing a controlled-release matrix containing INH has been reported to reduce the release rate of INH and minimize the initial burst as well as the sample variability.[4] PPF has been explored as a component of bone cement. Gerhart et al.[14] formulated a biodegradable cement using methyl methacrylate monomer to crosslink the unsaturated PPF. Wise et al.[15] further used PPF with vinyl pyrrolidone (VP) as a crosslinking agent to form a bone cement. Sanderson[12] reported the use of a PPF–VP cement for controlled drug release and showed that release from these crosslinked PPF matrices is controlled by surface erosion rather than by diffusion. This release mechanism is contrary to the release mechanism from PLGA matrices, in which diffusion controls the release. As PPF, an unsaturated polyester, and PBLG, a polyamide, are expected to have different degradation rates and polymer crystallinity, it is interesting to compare kinetics of release of a model compound such as INH. Two solvents, glacial acetic acid and benzene, were selected for preparation of foams by lyophilization. These solvents have relatively high melting points as well as high vapor pressures near their melting points, and thus are favorable candidates for lyophilization (Table I).

While benzene readily dissolved PLGAs, PPF, and PBLG, it did not dissolve PLLA or PGA. However, PLLA was observed as soluble in a hot glacial acetic acid solution at 96°C. Only the PLGAs and PPF were soluble in glacial acetic acid. The result is shown in Table II.

TABLE I

	Melting Point[16]	Vapor Pressure[16]
Glacial acetic acid	16.7°C	10 mm Hg at 17.5°C
Benzene	5.5°C	40 mm Hg at 7.6°C

The structures of the resulting polymeric foams, prior to the drug impregnation and compaction, were examined by SEM and are shown in Figures 1–4. As can be seen from these SEMs, the morphology of each polymer foam shows different porous structures, depending on the polymer and solvent used for foam preparation. Generally, PLGA foams prepared from glacial acetic acid have leaflet or platelet structures of more or less parallel thin films, while PLGA foams prepared from benzene display capillary structures. PBLG yielded a leaflet structure from benzene. These structures can be easily identified in Figures 1 and 2, which show PLGA-85:15 foams prepared from glacial acetic acid and benzene as solvents, respectively. PBLG with M_w = 150,000 was lyophilized to form a foam from a benzene solution. Its structure, shown in Figure 3, is characterized by leaflets rather than by the capillaries observed in PLGA–benzene foams (Fig. 2). This indicates that the foam structure is determined not only by the solvent but also by the polymer. The attempt to make PPF foams did not succeed; PPF particles remained after the solvent was totally removed. This suggests that the low-molecular-weight PPF (M_w = 7600) cannot provide sufficient strength to support a foam structure. This result implies a low-end limit in molecular weight in this polymeric foam fabrication method. Nevertheless, a polymer composite foam containing 50 wt % of PLGA-85:15 and 50 wt % of PPF was successfully made by lyophilization of a glacial acetic acid solution containing both polymers. Its leaflet-like morphology is shown in Figure 4.

The results of other foam characterization, including foam density, molecular weight, and glass transition

TABLE II
Dissolutions of Polymers in Solvents

Polymers	Glacial Acetic Acid	Benzene
PLLA	–*	–
PLGA-85:15	+	+
PLGA-75:25	+	+
PLGA-58:42	+	+
PGA	–	–
PPF	+	+
PBLG	–	+

*PLAA was soluble in glacial acetic acid at 96°C. +: soluble; –: insoluble.

Figure 1. Scanning electron micrograph of PLGA-85;15 foam prepared by lyophilization of glacial acetic acid solution, 10 KV. Original magnification ×100.

temperature, are summarized in Table III. These foam characteristics are compared with the characteristics of the as-received polymers prior to foam formation. Thermal analyses of the foams were conducted by differential scanning calorimetry (DSC) to determine their T_g's. The results indicate that PLGA-85:15, PPF, and their mixture, the PLGA–PPF foam, were still amorphous and showed single glass transition temperatures of 59.3°C, 30.7°C, and 54.3°C, respectively. In contrast, PBLG showed no detectable glass transition peak in the thermal graph, indicating that PBLG is more crystalline than the others. The glass transition temperatures before and after foam formation are considered to be unchanged for each foam. The observation of a single T_g rather than dual T_g's in the thermal graph indicates that the PLGA–PPF foam was a homogeneous mixture.

The resulting foam densities were always observed to be higher than their polymer concentrations, i.e., the foam volumes after lyophilization shrank to less than the original volumes of the polymer solutions. To compare the foam shrinkages for the polymers used in this study, a polymer concentration, 45 mg/mL, was selected to prepare the low-density foams. This concentration yielded foam densities ranging from 51.3 to 77.6 mg/cm^3 depending on the polymers (Table III). This shrinkage is considered to result from the molecular rearrangement which occurs during lyophilization. In an amorphous polymer, the polymer chains are distributed in a completely random manner, and thus possess greater mobility than crystalline polymer chains. During lyophilization, the chains of an amorphous polymer would have a greater chance than those of a crystalline polymer to move and re-form when the holding solvent is removed, and thus tend to approach a minimum volume. This is evidenced by the aggravated shrinkage occurring in amorphous polymer foam formations. To show this effect, we have calculated the ratios of the foam densities to their original polymer concentrations and reported the values in Table III. Obviously, the crystalline PBLG has the highest ratio of foam density to polymer solution concentration (0.889); that is, PBLG shows least shrinkage during foam formation. On the other hand, amorphous PLGA–PPF has the lowest value of that ratio (0.615), indicating that shrinking effect is maximal for this amorphous polymer.

The molecular weights before and after foam formation were essentially unchanged within experimental error. Based on this GPC result and the observation of unchanged T_g's after foam formation, it can be assumed that neither solvent caused polymer degradation.

We have shown in related work that entrapment of INH microcrystals between polymer leaflets prior to matrix extrusion would minimize release through channels along grain boundaries while providing a more continuous lattice through which diffusion of INH would have to occur.[4] Based on this result, we prepared several controlled-release matrices using the polymer foams described in the previous section for *in vitro* evaluation of INH release. PLGA-85:15 foams were prepared from both benzene and glacial acetic acid. INH loadings in the

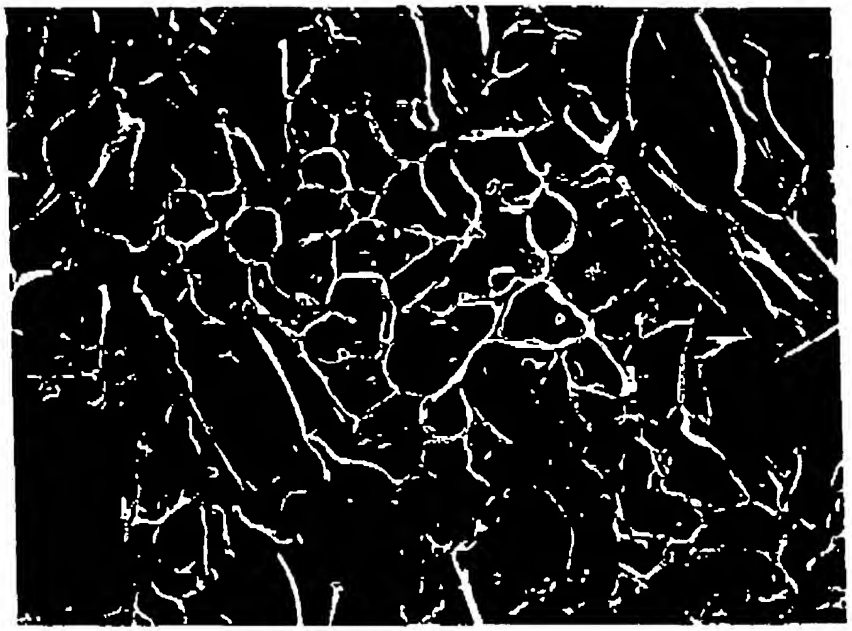

Figure 2. Scanning electron micrograph of PLGA-85:15 foam prepared by lyophilization of benzene solution, 10 KV. Original magnification ×100.

final matrix varied only from 18.3 to 21.5 wt %. Thus, we were able to examine the effects of foam structure and density for several polymers on INH release. The characteristics of these matrices are summarized in Table IV.

In vitro release profiles are shown in Figures 5–8. Several features immediately apparent are listed below. Numbers in parentheses are matrix identifications.

1. Foam density has a significant influence on the release rate. Figure 5 shows that the matrices pre-

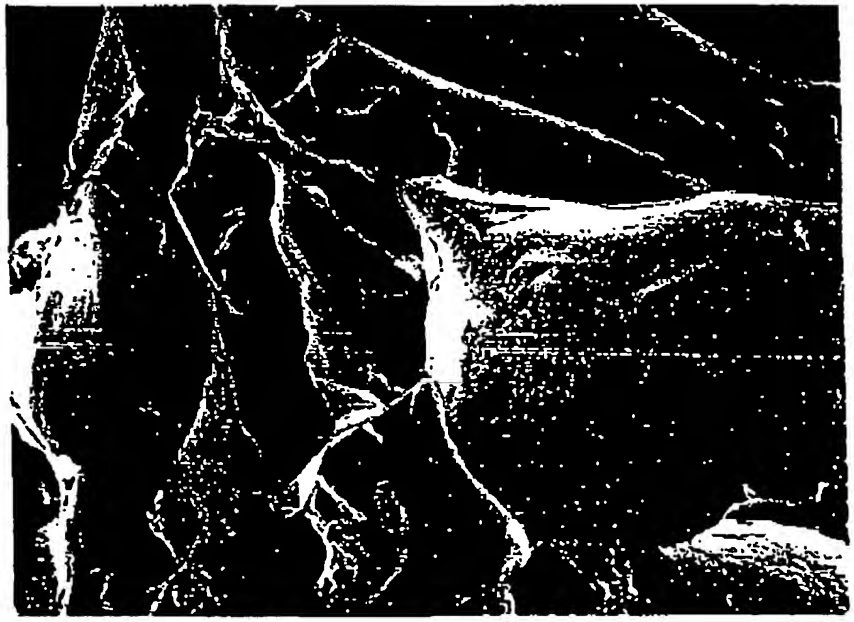

Figure 3. Scanning electron micrograph of PBLG foam prepared by lyophilization of benzene solution, 10 KV. Original

Figure 4. Scanning electron micrograph of PLGA-85:15/PPF (50/50 wt. %) foam prepared by lyophilization of glacial acetic acid solution. 10 KV. Original magnification ×100.

pared from lower-density foams release INH more rapidly than from higher-density foams.

2. The matrix (65–62), prepared from the composite foam of PLGA and PPF, released much faster than the matrix prepared from PLGA-85:15 foam (32-79). This suggests that the effect of faster release is likely attributable to the lower-molecular-weight PPF in the composite foam, which usually allowed greater absorption of water into the matrices to diffuse[17] (Fig. 6).
3. The selection of a solvent in making foams does not show a pronounced effect on the release. We compared the release rates from two PLGA matrices prepared from glacial acetic acid foam (32–79) and benzene foam (65–63), and found that the release from the benzene foam was not significantly different from that from the glacial acetic acid foam (Fig. 7) as determined by statistical analysis using the methods of Tukey.[18]
4. INH released from the PBLG foam matrix (65–64) was compared with its release from PLGA matrix (65–63). Figure 8 shows that the PLGA foam matrix released more rapidly than PBLG foam matrix. This is in accord with expectations. PBLG is a crystalline polymer. The transport of INH molecules through a crystalline polymer is usually restricted because the transport proceeds mainly via the amorphous regions. This is analogous to the permeability as observed in membrane separation technology. The permeability strongly de-

TABLE III
Characteristics of Foams and Their Originating Polymers

Polymer or Foam	Polymer Conc. (mg/mL) (a)	Foam Density (mg/cm³) (b)	Ratio of Shrinkage (a/b)	T_g (°C)	M_w*
PLGA-85:15†				59.3	179,000
PLGA (GHAc)‡	51.3	70.7	0.731	60.4	166,000
PLGA (Benz)§	52.5	77.6	0.676	59.4	170,000
PPF¶				30.7	7600
PLGA/PPF#	45.2	73.5	0.615	54.3	
PBLG**				None	150,000
PBLG (Benz)††	45.6	51.3	0.889	None	150,000

*Weight average molecular weight.
†Polymer used for foam formation.
‡PLGA-85:15 glacial acetic acid foam.
§PLGA-85:15 benzene foam.
¶PLGA-85:15–PPF (50/50 wt. %) glacial acetic acid foam.
**PBLG benzene foam.

TABLE IV
Description of Matrices

Matrix ID*	Polymer	Solvent	Foam Density (mg/cm³)	INH Aq. Conc. (mg/mL)†	Loading wt. %
32–79	PLGA-85:15	GHAc‡	70.70 ± 1.45	16.5	18.31
65–60	PLGA-85:15	GHAc	44.28 ± 1.21	12.0	21.05
65–61	PLGA-85:15	GHAc	85.89 ± 1.44	21.5	19.04
65–62	PLGA–PPF	GHAc	73.48 ± 1.30	20.0	21.45
65–63	PLGA-85:15	Benzene	77.64 ± 2.15	20.0	18.99
65–64	PBLG	Benzene	26.20 ± 4.64	6.5	19.46
65–65	PLGA-85:15	Benzene	35.69 ± 3.62	10.0	20.64

*Rod diameters varied from 1.66 to 1.70 mm (see Table V).
†INH aqueous concentrations used to obtain the final INH loading in matrices.
‡Glacial acetic acid.

pends on the degree of crystallinity of the membrane.

Release kinetics were analyzed by the Roseman–Higuchi diffusion model which had been confirmed to fit the INH release kinetics from INH–PLGA matrices.[4,11] The cumulative fraction released versus time curves may be linearized by plotting data as

$$(1-F)\ln(1-F)+F=\frac{4CDt}{Aa_o^2} \qquad (4)$$

where

F = cumulative fraction released at time t, days

C = solubility of drug in the polymer, mg/cm³

D = diffusivity of the drug in the polymer, cm²/day

A = concentration of INH in the matrix, mg/cm³

a_o = rod radius, cm

The correlation coefficients (R^2) for the linear regression and the diffusivities calculated from the slopes are given in Table V.

The linearity confirms that the release kinetics for the foam-based matrices fits the Roseman–Higuchi diffusion model, implying that the release mechanism is diffusion controlled. The resulting INH diffusivities

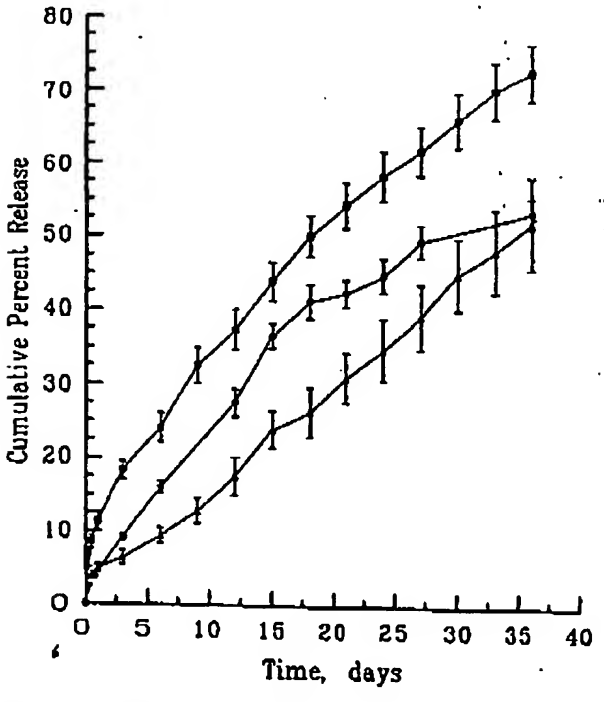

Figure 5. Effect of foam density on cumulative release of INH: matrix 32–79 (●) (ρ_f = 70.7 cm³); matrix 65–60 (■) (ρ_f = 44.3 mg/cm³; matrix 65–61 (▲ (ρ_f = 85.9 mg/cm³).

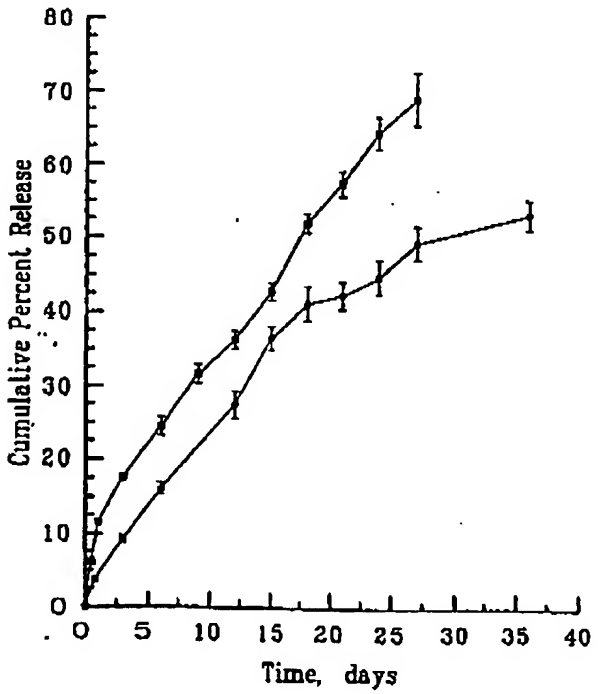

Figure 6. Effect of polymer on cumulative release of INH; matrix 32–79 (●) (PLGA-85:15 foam); matrix 65–62 (■) (PLGA–PPF foam).

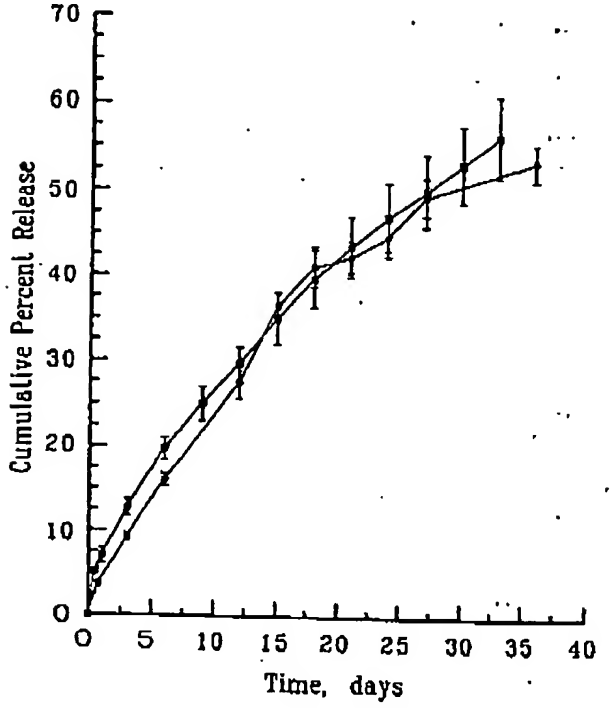

Figure 7. Effect of solvent on cumulative release of INH; matrix 32–79 (■) (glacial acetic acid); matrix 65–63 (●) (benzene).

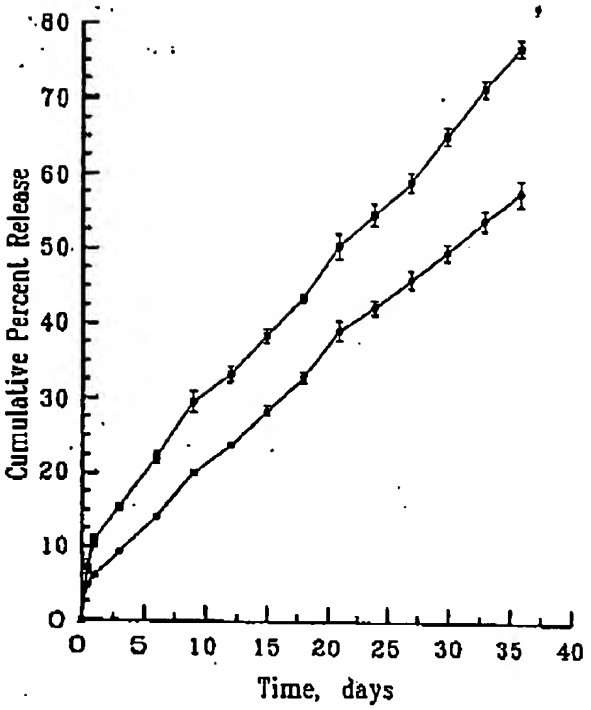

Figure 8. Effect of polymer on cumulative release of INH; matrix 65–64 (■) (PLGA-85:15 foam); matrix 65–65 (●) (PBLG foam).

range from 0.99–2.59 × 10^{-4} cm^2/day. It is interesting to note that these diffusivities are between those calculated for INH release (or for a molecule of that molecular weight) from natural rubber (1.98 × 10^{-3} cm^2/day) and from polystyrene (4.30 × 10^{-8} cm^2/day).[19] This indicates that the INH release from the foam-based matrices is controlled by drug diffusion rather than by polymer degradation. (Otherwise, the calculated diffusivities for INH release from the matrices should be greater than those from nondegradable natural rubber or polystyrene.)

CONCLUSIONS

A fabrication method for preparing porous biopolymers is described. The method, using either glacial acetic acid or benzene as solvent, can successfully produce porous, low-density polymeric foams of several biopolymers, including PLGA, PLGA–PPF, and PBLG. The structures of these porous foams are generally classified into two categories: leaflet and capillary, depending on the polymer and solvent used in fabrication. *In vitro* release of INH from the matrices prepared from PLGA-85:15, PLGA–PPF, and PBLG foams shows significant differences in release kinetics. The INH owing to the low-molecular-weight PPF which appears to compromise the integrity of the foams. Contrary to PLGA–PPF foam matrix, PBLG foam matrix has the slowest release because of high crystallinity. PLGA-85:15 foam matrix has the medium release rate between PLGA–PPF matrix and PBLG matrix. The density of the polymer foam has a significant influence on the release rate: Higher-density foams result in slower release. It is concluded that the release mechanism of the foam matrices is lattice diffusion controlled.

TABLE V
Results of *In Vitro* Release Measurements

Matrix ID[a]	Slope K day^{-1}	R^2[b]	Rod Diameter (mm)	[INH] $(mg/cm^3)^2$	D (cm^2/day)
32–79	0.00541	0.98	1.67	183.1	1.15 × 10^{-4}
65–60	0.01036	0.98	1.69	210.5	2.59 × 10^{-4}
65–61	0.00433	0.91	1.70	190.4	0.99 × 10^{-4}
65–62	0.01156	0.94	1.68	214.5	2.92 × 10^{-4}
65–63	0.00609	0.99	1.69	189.9	1.38 × 10^{-4}
65–64	0.00572	0.95	1.66	194.6	1.28 × 10^{-4}
65–65	0.01081	0.93	1.67	206.4	2.59 × 10^{-4}

[a]Three replicates were run for each matrix.
[b]Correlation coefficient for linear regression of $(1 - F)\ln(1 - F) + F$ versus time.

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Mucosal immunization with a measles virus CTL epitope encapsulated in biodegradable PLG microparticles

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Abstract

The immunogenicity of a cytotoxic T cell epitope (CTL) representing residues 52–60 from measles virus (MV) nucleoprotein, encapsulated in poly(lactide-co-glycolide) (PLG) microparticles was evaluated after mucosal immunization. After intranasal administration of the encapsulated CTL epitope linked at the carboxyl terminus of two copies of a T-helper epitope (TT-NP6), peptide-specific and MV-specific CTL responses were detected in splenocytes. However, these responses were lower than the responses observed when the TT-NP6 peptide was administered intranasally in saline or using CTB as an adjuvant. Intranasal coadministration of the encapsulated TT-NP6 peptide with CTB did not result in any significant potentiation of the CTL responses. The effectiveness of biodegradable PLG microparticles for mucosal delivery of CTL epitopes, combined with their excellent tissue compatibility and biodegradability suggests that they represent a valuable delivery system for synthetic immunogens. However, further work is needed to define the requirements for effective absorption by the nasal epithelium.

Keywords: Cytotoxic T cell epitope; Intranasal immunization; Measles virus; Poly(lactide-co-glycolide) microparticle

1. Introduction

Although most human pathogens gain entrance via the mucosal surfaces, the majority of the currently available vaccines have been developed for systemic immunization. To a large extent this reflects the relative difficulty in achieving effective mucosal immunization. Numerous studies in animals and humans have provided convincing evidence that protection against a variety of viral and bacterial mucosal pathogens can be obtained by oral or intranasal vaccination (Moldoveanu et al., 1993; Tamura et al., 1992). This has resulted in an intense search for safe and immunogenic mucosal vaccines. However, a major problem has been the poor bioavailability of antigens delivered mucosally, due to proteolytic degradation. For this reason much attention has been directed towards the development

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of delivery systems for mucosal vaccination and PLG microparticles have been considered a candidate for mucosal vaccine delivery. Unlike soluble antigen, mucosally administered antigen entrapped in PLG microparticles can induce good local and systemic antibody responses (Eldridge et al., 1991; Maloy et al., 1994). However, the ability of this delivery system to induce CTL responses has not been extensively studied. Intraperitoneal delivery of PLG encapsulated MV CTL epitopes induces long term peptide- and MV-specific CTL responses (Partidos et al., 1996a). In this report, we describe the potential of PLG microparticles for the administration of peptides representing MV CTL epitopes via the intranasal route.

2. Materials and methods

The NP6 CTL epitope from MV nucleoprotein (NP), representing residues 52–60 (LDRLVRLIG) (NP6) (Beanverger et al., 1994) was co-linearly synthesized using Fmoc chemistry at the carboxyl terminus of two copies (TT-NP6) of a T-helper epitope (T) representing residues 288–302 from the fusion protein of MV (Partidos and Steward, 1990; Partidos et al., 1996b). The chimeric peptide was encapsulated in PLG microparticles by a solvent extraction method as described previously (Jones et al., 1995). The mean diameter of the microparticles was 1.88 μm and the efficiency of encapsulation was 30%.

The immunogenicity of TT-NP6 peptide was tested after intranasal administration of groups of three CBA mice (6–8 weeks old) with 50 μg/dose of peptide either encapsulated (group A), free in saline (group B) or coadministered with cholera toxin B subunit (CTB) (Sigma, 10 μg/dose) free peptide in saline (group C) or encapsulated peptide (group D) in 30 μl volume of phosphate buffer saline on 3 consecutive days. Mice were boosted 3 weeks later with a single dose of 50 μg/dose of peptide encapsulated (group A), free in saline (group B) or coadministered with CTB (10 μg/dose) free peptide in saline (group C) or encapsulated peptide (group D).

Two weeks after intranasal administration, immune splenocytes were harvested and restimulated in vitro with the NP6 peptide for 7 days. On day 3, 10% (v/v) rat Con A supernatant (as a source of IL-2) was added to the cultures. CTL activity was assessed using the ^{51}Cr release assay as described previously (Partidos et al., 1996b).

3. Results and discussion

The potential of the intranasal route for mucosal immunization was tested with the encapsulated TT-NP6 chimeric peptide. As shown in Fig. 1, effector splenocytes from mice immunized with the TT-NP6 peptide with CTB as an adjuvant or free in saline could lyse L929 (panel a) or NS20Y (panel b) (Rager-Zisman et al., 1984; Gopas et al., 1992) target cells pulsed with 1 μM of NP6 peptide or persistently infected with MV (panel c) more effectively than effector cells from mice immunized with encapsulated TT-NP6 peptide. No significant lysis was observed in non-pulsed target cells. Furthermore, when mice were immunized intranasally with encapsulated TT-NP6 peptide coadministered with CTB, no significant potentiation of CTL lysis was observed to either L929 (Fig. 2a), NS20Y (Fig. 2b) target cells pulsed with 1 μM of NP6 peptide or to NS20Y/MS persistently infected with MV target cells (Fig. 2b).

For the development of synthetic peptide immunogens with the potential to induce CTL responses, the peptides must have the ability to insert into the cell membrane of antigen presenting cells (APCs) and facilitate processing via the class I pathway. The finding that PLG microparticles can act as a delivery system for CTL epitopes suggests that after being taken up by APCs they can escape into the cytoplasm where the released CTL epitope could then be transported to the endoplasmic reticulum by a cytoplasmic peptide transporter (Yewdell and Bennick, 1990; Discoll and Finley, 1992). Thus, the CTL epitope gains access to the pathway for class I presentation. Studies by Eldridge et al. (1991) have suggested that the predominant mechanism of immune enhancement by microparticles is the direct intracellular delivery to accessory cells of high concentrations of antigen incorporated within the copolymer matrix. Indeed, microparticles can be rapidly phagocytosed by macrophages (Tabata and Ikada, 1990) which can serve as APCs for the generation of CTLs (Debrick et al., 1991).

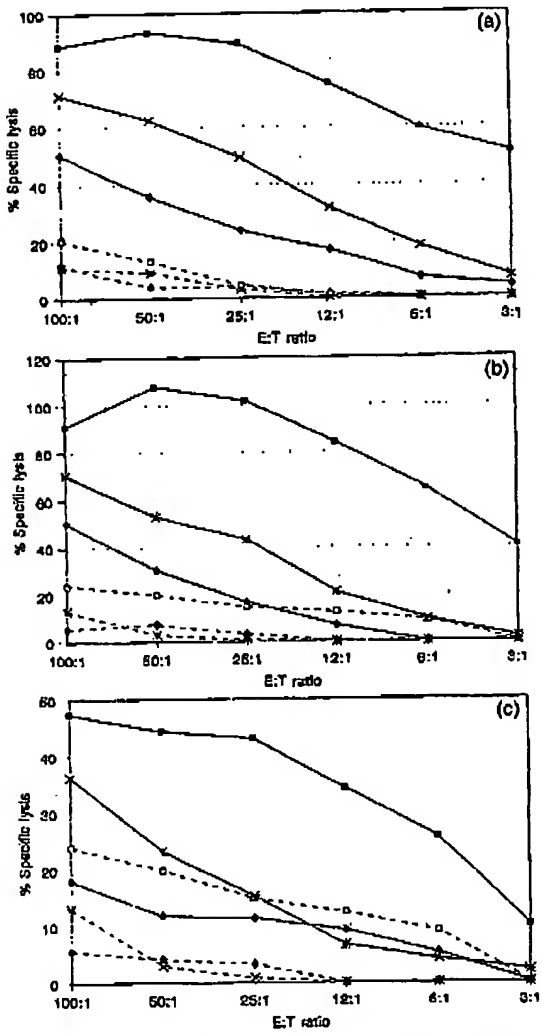

Fig. 1. CTL activity induced after intranasal priming of groups of CBA mice with the TT-NP6 peptide administered free in saline (*), encapsulated in PLG microparticles (♦) or coadministered with CTB (■). After in vitro restimulation of splenocytes with NP6 peptide, effector cells were cocultured with L929 (*a*), NS20Y (*b*) target cells pulsed (————) or non-pulsed (– – –) with 1 μM of NP6 peptide or the persistently infected MV NS20Y/MS cell line (————) (*c*) at the indicated E:T ratio. Spontaneous release was 12% for L929 cells and 24% for NS20Y and NS20Y/MS cells of the total release by detergent in all assays. Data represent the mean of triplicates for each E:T ratio after pooling the spleen cells from groups of three mice. The SD of triplicate wells was consistently less than 10% of the mean.

The effectiveness of PLG microparticles as a delivery system is further highlighted by their ability to induce CTL responses after intranasal immunization. Successful induction of systemic and mucosal antibody responses after mucosal administration of PLG microparticles has been reported for several microencapsulated antigens (Moldoveanu et al., 1993; Eldridge et al., 1991) and in recent reports PLG microparticles have been shown to prime for ovalbumin (OVA)-specific CTL responses after parenteral and oral immunization (Maloy et al., 1994) and HIV-specific CTL responses after intranasal pri-

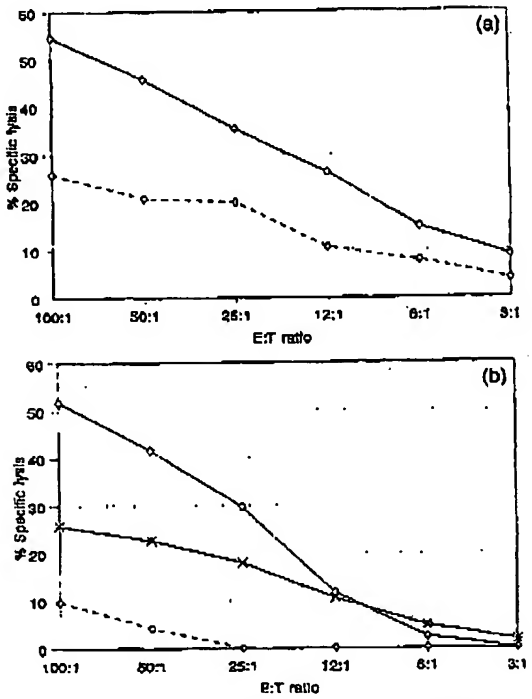

Fig. 2. CTL activity induced after intranasal administration in a group of three CBA mice of encapsulated TT-NP6 peptide with CTB. After in vitro restimulation of splenocytes with NP6 peptide, effector cells were cocultured with L929 (*a*), NS20Y (*b*) target cells pulsed (————) or non-pulsed (– – –) with 1 μM of NP6 peptide or with the persistently infected MV NS20Y/MS cell line (*b*) (* ———— *) at the indicated E:T ratio. Spontaneous release was 10.58% for L929 cells and 28% for NS20Y and NS20/MS cells of the total release by detergent in all assays. Data represent the mean of triplicates for each E:T ratio after pooling the spleen cells from a group of three mice. The SD of triplicate wells was consistently less than 10% of the mean.

minig with encapsulated gp120 protein (Moore et al., 1995). The results presented here are consistent with these findings and demonstrate for the first time the potential of PLG microparticles for the delivery of synthetic peptides representing CTL epitopes via the intranasal route. However, the responses with the encapsulated peptide were lower than the responses observed after intranasal administration with free peptide in saline or free peptide coadministered with CTB. This suggests that the microparticles might not be absorbed efficiently by the nasal epithelium. It has been suggested that soluble antigens may easily penetrate the whole nasal epithelium as compared to particulate antigens which may be removed quickly from the nasal mucosa by the mucociliary system (Kuper et al., 1992). Moreover, although CTB has been suggested to exert its adjuvanticity by enhancing the transepithelial influx of the vaccine into the nasal mucosa where the immunocompetent cells are located (Gizurarson et al., 1992), its coadministration with the encapsulated peptide did not result in any significant potentiation of CTL responses. Thus, although it appears that biodegradable PLG microparticles can act as an effective delivery system for mucosal immunization of synthetic peptides representing viral CTL epitopes, further work is needed to define the requirements for more effective absorption by the nasal epithelium.

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Gene gun-based nucleic acid immunization: elicitation of humoral and cytotoxic T lymphocyte responses following epidermal delivery of nanogram quantities of DNA

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Particle-mediated (gene gun) DNA transfer to the epidermis was evaluated for its ability to elicit humoral and cytotoxic T lymphocyte responses using decreasing quantities of plasmid DNA-based antigen expression vectors. Using plasmids encoding human growth hormone, human alpha-1-antitrypsin, and influenza virus nucleoprotein, strong immune responses were observed in mice following immunization with as little as 16 ng of DNA using an electric discharge gene delivery system. Significant antibody titers were observed against these antigens following a primary immunization, with responses rising dramatically following a boost. Increasing the DNA dose above 16 ng per immunization had little beneficial effect. In contrast to particle-mediated DNA delivery, intramuscular or intradermal inoculation required greater than 5000-fold more DNA to achieve comparable results. Data are also presented demonstrating that a simple, hand-held version of the Accell™ *DNA delivery system, employing compressed helium as the particle motive force, achieves immune responses comparable to the traditional electric discharge device.*

Keywords: Gene gun; DNA immunization; influenza; skin

Nucleic acid immunization involves the direct *in vivo* administration of antigen-encoding expression vectors for the purpose of eliciting antigen production and resultant specific immune responses[1-8]. This technology mimics live attenuated vaccines in that antigens are produced in their native conformation and are presented in the context of MHC class I and class II molecules to elicit cytotoxic cellular and humoral immune responses, respectively. This report is an extension of the study of Fynan *et al.*[4], in which it was demonstrated that particle-mediated (gene gun) delivery of an influenza virus hemagglutinin expression vector to the epidermis was superior to intramuscular inoculation for the elicitation of protective immunity in mice. Using three new antigen expression vectors, we demonstrate that the *Accell*™ particle-mediated gene delivery system[1,2] elicits primary IgG responses following a single immunization with as little as 16 ng of plasmid DNA and that the respective titers can be boosted by 5- to 10-fold following a second immunization. The induction of similar responses via intramuscular inoculation required >5000-fold more DNA. We show that this difference in efficacy is likely due to the method rather than the site of delivery since intradermal inoculations also required >5000-fold more DNA to approach the titers obtained following particle-based DNA immunization of the skin. Finally, we demonstrate that a simple, hand-held gene delivery instrument that uses compressed helium as the particle motive force can achieve immunological results similar to those obtained using the more traditional and complex electric discharge device.

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MATERIALS AND METHODS

Expression vectors

pCMV-hGH contains the human cytomegalovirus (hCMV) immediate early promoter and encodes human growth hormone[1]. pCMV-hAAT encodes human alpha-1-antitrypsin (hAAT) and was constructed by inserting the 1.4 kb Not I fragment derived from pKP1-hAAT (Dr Kathy Ponder, Washington University, St. Louis, MO) into the pCMVβ vector (Clonetech, Palo Alto, CA)

following digestion with Not I to remove the beta-galactosidase gene. pCMV-NP was a gift of Dr Kari Irvine, National Cancer Institute, and contains the complete nucleoprotein (NP) coding sequence from influenza virus A/PR/8/34.

Particle-mediated DNA immunizations

Plasmid DNAs were accelerated into the abdominal epidermis of 6–8-week-old female BALB/c mice using the electronic *Accell*™ gene delivery system (Agracetus Inc., Middleton, WI) as previously described[1,2,4], except that the skin was not pretreated in any way except for the removal of fur in the local area using clippers. All immunizations utilized a delivery energy of 15 kV. Epidermal immunizations employing a hand-held, helium-powered *Accell*™ instrument contained 0.5 μg of DNA and 0.5 mg of 0.95 micron gold powder using a helium pressure setting of 400 p.s.i. The instrument is described in a recent PCT patent application[9].

Intramuscular and intradermal DNA inoculations

All intramuscular DNA immunizations involved injection of the quadriceps with 0.05 ml of 0.9% saline containing from 1 to 100 μg of plasmid DNA. All immunizations were administered invasively to anesthetized mice to guarantee proper placement of the inoculum. After anaesthetizing 6–8-week-old female BALB/c mice[1,2], a 1.5 cm incision was made through the skin along the inner thigh to expose the leg muscle groups. This was followed by injection of the DNA solution into the quadriceps and subsequent closure of the incision with surgical staples. Intradermal DNA immunizations were as described[8].

Antibody titer determination

Collection of blood samples and antibody titer determinations were as described previously[1,2]. For titration of influenza nucleoprotein IgG samples, 96-well plates were coated with detergent-disrupted influenza virus (50 μl per well of virus strain A/PR/8/34). In this case, sufficient virus was disrupted in lysis buffer (0.5 M Tris–HCl (pH 7.8), 0.6 M KCl, 0.5% Triton X-100) for 5 min at room temperature and then diluted with PBS to a final concentration of 4000 HA units per ml.

Cytotoxic T lymphocyte assays

Cytotoxic T lymphocyte responses to influenza nucleoprotein were measured as previously described[2] except that the synthetic nucleoprotein peptide (TYQRTRALV)[10] was substituted for the HIV-1 gp120 peptide.

RESULTS AND DISCUSSION

Figure 1 shows the results of two immunization trials comparing the endpoint IgG titers elicited to human growth hormone (hGH) and human α-1-antitrypsin (hAAT), respectively, following intramuscular (injection) or epidermal (gene gun) DNA immunizations using three different doses of DNA. In all cases, geometric mean titers following a single immunization were

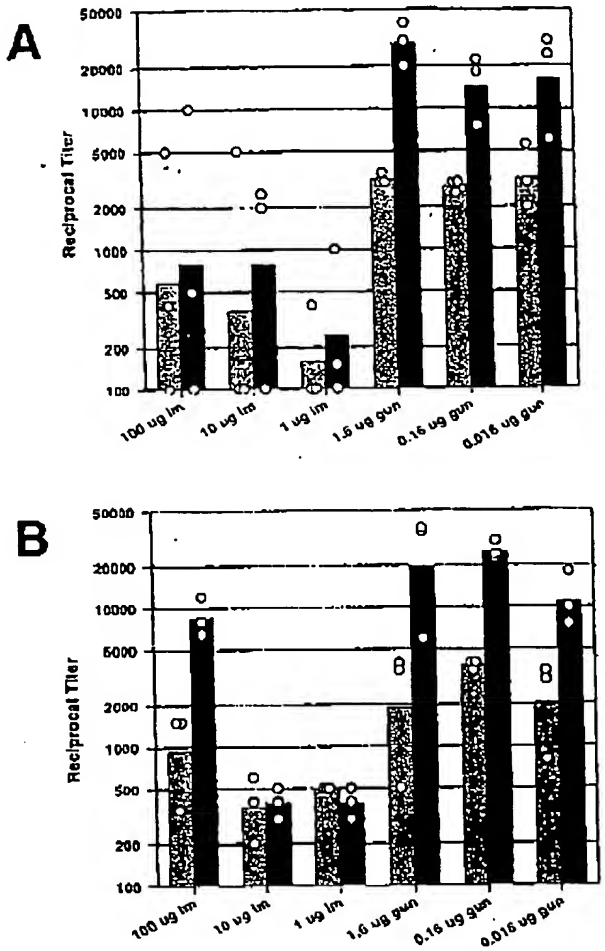

Figure 1 Six groups of three female BALB/c mice were each immunized on days 0 and 28 with the indicated amount of either pCMV-hGH or pCMV-hAAT DNA, either by intramuscular inoculation or via particle-mediated DNA delivery (gene gun) to the abdominal epidermis. Serum samples were collected on days 28 and 42 to measure primary and booster responses, respectively. Panel A, pCMV-hGH immunizations; Panel B, pCMV-hAAT immunizations. Gray bars, geometric mean titers following the primary immunization; solid bars, geometric mean titers following the booster immunization. Open circles show titers of individual mice

highest in the gene gun-immunized animals and the titers obtained were independent of the amount of DNA employed (1.6–0.016 μg DNA per immunization). The efficacy of the gene gun immunizations was further enhanced following a booster immunization in which the titers of all groups increased by 5- to 10-fold. In contrast, geometric mean titers in the intramuscular groups were considerably lower except for the group that received the 100 μg hAAT DNA immunizations. In the latter case, the responses were similar to those observed in the gene gun groups that received as little as 16 ng of DNA per immunization.

To determine if the enhanced immune responses following particle-mediated, intracellular delivery were due to the method rather than the site of delivery, a similar dose titration study was performed using an influenza nucleoprotein (NP) vector and substituting intradermal inoculation[8] for the more traditional intramuscular injection approach. IgG and cytotoxic T lymphocyte

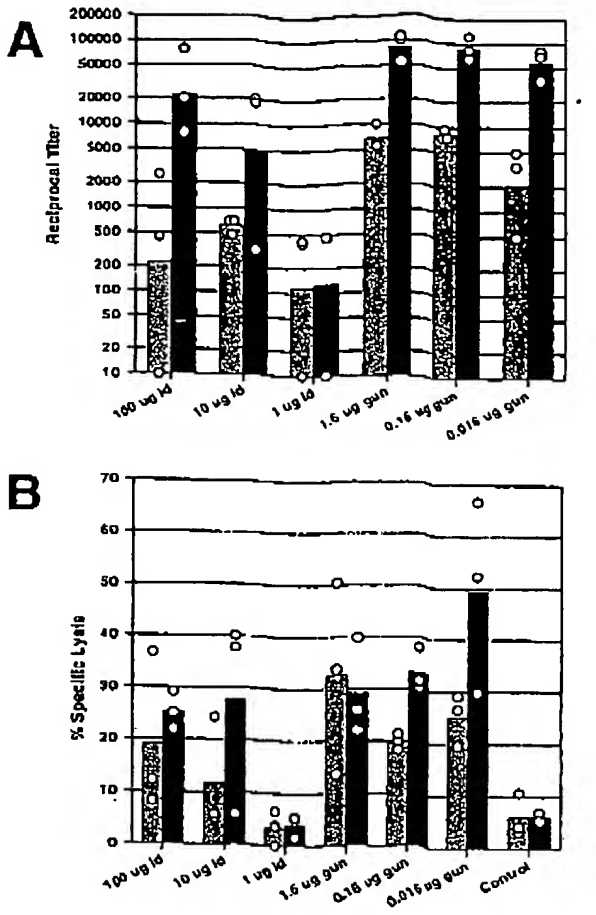

Figure 2 Six groups of three female BALB/c mice were each immunized on day 0 and another six groups were immunized on days 0 and 28 with the indicated amounts of pCMV-NP DNA, either by intradermal inoculation or via particle-mediated DNA delivery (gene gun) to the abdominal epidermis. Serum samples and splenocytes were collected on day 28 for the animals that received only a single immunization. Serum samples and splenocytes were collected on day 42 for the animals that received two immunizations. Panel A, IgG responses to NP (gray bars, geometric mean titers following primary immunization; solid bars, geometric mean titers following two immunizations). Panel B, CTL responses to NP (gray bars, average percent lysis following primary immunization; solid bars, average percent lysis following two immunizations). All effector-to-target ratios were 25:1. Animals marked "control" were immunized with irrelevant DNA

responses from this experiment are shown in *Figure 2*, Panels A and B, respectively. Similar to the previous comparisons, the strongest responses were observed in the gene gun-immunized animals with little evidence for a decrease in efficacy following reduction of the dose to as little as 16 ng of DNA per immunization. In contrast, geometric mean titers in the intradermally injected animals were not as pronounced, even following injection of as much as 100 μg of DNA. The cytotoxic T lymphocyte responses in these same animals were consistent with the IgG results in that dosage effects were not observed in the gene gun-immunized groups, but were seen following intradermal inoculation (*Figure 2b*). Control animals indicated in *Figure 2b* were immunized with irrelevant DNA. Additional controls in the CTL assay included coating target cells with irrelevant peptide in which background lysis values of 10% or less were

Table 1 NP-specific IgG titers following particle-mediated immunization with pCMV-NP DNA using the electric discharge and helium pulse instruments. Non-immunized control animals exhibited IgG titers of less than 1:10 (not shown)

Instrument	4 weeks post-prime	6 weeks post-boost
Helium pulse	4400	50 000
	5300	38 000
	3300	44 000
Electric discharge	4300	27 000
	3600	27 000
	4500	40 000

observed (not shown). NP-specific lytic activity was also shown to be associated with the $CD8^+$ T cell fraction following fractionation of lymphocyte subsets (not shown).

While this report and others have indicated that particle-mediated DNA immunization is an effective means of eliciting humoral and cytotoxic T lymphocyte responses in animal models, it has traditionally required an electronic instrument that is not practical for widespread clinical use. However, recent advances in gene gun technology have resulted in the development of a simple, inexpensive, hand-held *Accell*™ DNA delivery device that is better suited for particle-mediated gene delivery in clinical settings[9]. The effectiveness of this instrument for administration of nucleic acid vaccines is shown in *Table 1* in which the immune responses to NP were compared in animals immunized with 0.5 μg of the NP vector using either the electric discharge or the helium pulse devices. Essentially identical responses were obtained in both groups of animals, demonstrating a potential clinical role for particle-based DNA delivery technology in the area of vaccination and immunomodulation.

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Controlled Delivery of Diphtheria Toxoid Using Biodegradable Poly(D,L-Lactide) Microcapsules

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Diphtheria toxoid, which is an important vaccine in the expanded program of immunization (EPI) in the developing countries, was microencapsulated using poly(D,L,-lactide) of 49,000 molecular weight and the in-water drying technique. The microcapsules were subjected to an *in vitro* antigen release study using a sensitive enzyme-linked immunosorbent assay (ELISA) developed in the laboratory. Antibody titers in immunized Balb/C mice were also determined using direct ELISA. The antibody units in the immunized group till day 75 were quite comparable to those in the group receiving conventional three-dose injection of diphtheria toxoid with calcium phosphate as an adjuvant. SEM photographs of the microcapsules during *in vitro* degradation demonstrated the erosion kinetics of the polymer, leading to controlled release of the antigen.

KEY WORDS: vaccine delivery; biodegradable microcapsules; antigen ELISA; erosion kinetics; antibody titers.

INTRODUCTION

Controlled-release technology has recently shifted its emphasis from low molecular weight drugs to high molecular weight macromolecules, because many of the future drugs will be of recombinant DNA origin having high molecular weights. This development has led to newer polymers with a greater degree of biocompatibility and reproducible degradation kinetics (1,2). Vaccines are an example requiring novel controlled-release technology (3–5). Most vaccines require two or three primary immunizations, followed by a booster for optimum immune response. If one injection of the immunization schedule is missed, it leads to manifold loss of effective antibody titers. According to WHO statistics, more than 30% of the patients do not return for the next injection at each time point of the immunization schedule. The impact of noncompliance is most severe in the third world countries, where more than a million children die each year from vaccine-preventable diseases.

Ideally one would like to see the development of a controlled-delivery system that would release two or three doses of the vaccine in a programmable manner at one "single contact point administration." Such one-time vaccination under an expanded program of immunization would reach a greater percentage of the target population and afford protective antibody titers.

Diphtheria toxoid (MW 62,000) was choosen as a model vaccine, as it is a common vaccine in the immunization schedule worldwide and the toxoid, being a denatured protein, presents few stability problems. Also, the immunogenicity of the toxoid is well recorded. Stability studies carried out at this institute on β-hcG-DT, an antifertility vaccine, has shown that the carrier DT is stable at 37°C for 12 months with no loss of immunogenicity either *in vitro* or *in vivo*.

In the present study, diphtheria toxoid (DT) was microencapsulated using biodegradable poly(D,L-lactide) polymer, and the *in vitro* release monitored using an enzyme-linked immunosorbent assay (ELISA). The immune response to the antigen was determined in Balb/C mice. The antibody titers, between a group receiving a conventional dose of DT with calcium phosphate as an adjuvant and a group receiving subdermally implanted microspheres, were compared.

MATERIALS AND METHODS

Materials

Diphtheria toxoid (MW 62,000) having a concentration of 3500 Lf/ml (limes flocculation, the International Unit for vaccines) and a protein concentration of 15 mg/ml, was obtained from Serum Institute of India, Pune. Poly(D,L-Lactide) was obtained from Birmingham Polymers Inc. (Birmingham, AL) and Boehringer Ingelheim (FRG). Dichloromethane, polyvinylpyrrolidone and polystyrene standards were obtained from Aldrich Chemical Company, Inc. (Madison, WI). D,L-Lactic acid was obtained from Sigma Chemical Company (St. Louis, MO). The other chemicals were obtained from commercial suppliers and were used as received.

Quantitative Estimation of Diphtheria Toxoid

Diphtheria toxoid (DT) was measured by an enzyme-linked immunosorbent assay (ELISA) to estimate *in vitro* release rates from microcapsules and *in vivo* antibody titers in mice (6–9). Antigen (DT) detection was carried out using polyclonal sera containing anti-DT antibodies raised in goats to estimate the amount of antigen being released in the dispersion medium by the microcapsules. The 96-well ELISA plate was coated with increasing concentrations of DT in 50 *mM* coating phosphate buffer of pH 7.4 (from 10 to 100 ng/well). The plate was incubated at 37°C for 1 hr and then washed with phosphate buffer saline (50 *mM*, pH 7.4) with 0.2% Tween 20 (washing buffer). Then 100 μl of the antiserum (diluted 1:800) was added to each well, and the plate again incubated at 37°C for 1 hr. After incubation the plate was again washed with washing buffer thrice at an interval of 5 min between each washing. One hundred microliters of the conjugate Prot°A horse radish peroxidase (dilution, 1:25,000) was added to each well and the plate kept for incubation at 37°C for 1 hr. After incubation the plate was washed with the washing buffer thrice, and 100 μl of the substrate (0.05% of *O*-phenylenediamine and 0.1% of hydrogen peroxide in citrate phosphate buffer) was added to each well. The plate was incubated at 37°C for 15 min and then the reaction in each well was stopped with the addition of 50 μl 5 *N* sulfuric acid. Absorbance was read at 492 nm on an ELISA plate reader (Eurogenetics, NV, Belgium). The ab-

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sorbance-vs-concentration plot was linear from 10 to 100 ng. Each unknown sample was run with a standard curve in duplicate.

For the determination of the antibody units *in vivo* the assay principle was the same, the only difference being the coating of fixed antigen concentration initially and adding varying dilutions of the standard and test antisera. The second antibody was not limiting in these estimations. The antibody units were calculated by multiplying the dilution of the test sample by its absorbance reading.

Polymer Synthesis

For the above study both presynthesized commercial polymers and polymers synthesized in our laboratory were used. Poly(D,L-lactide) was synthesized using 160 g D,L-lactic acid monomers and 6 g activated ion-exchange resin (Dowex). The polycondensation reaction was performed at 185°C for 8 hr under vacuum and constant stirring. The resultant polymer had a low molecular weight (6000) as determined by gel permeation chromatography (GPC). A Waters GPC system was used with Ultrastrygel coulmns and a refractive index detector. The eluent used was tetrahydrofuran (THF) at 30°C and a flow rate of 1.0 ml/min. The commercial presynthesized polymers were also subjected to molecular weight determination, in comparison with standard polystyrene samples in THF. Poly(D,L-lactide) obtained from Birmingham Polymers, having a viscosity of 0.75 dl/g and a molecular weight of 49,000, was also used because the low molecular weight of the synthesized polymers made them unsuitable for long-term release study.

Microencapsulation

The vaccine was microencapsulated using the in-water drying method (10–13). To 1 ml of PBS (50 m*M*, pH 7.4), 150 Lf units of the vaccine and 100 mg of gelatin were added. Poly(D,L,-lactide) (1 g) was dissolved in 10 ml dicholoromethane. This organic solution was gradually added to the aqueous phase containing the vaccine with high-speed mixing on a ultrasonicator to form a fine emulsion. The temperature was lowered to 10°C by keeping the emulsion in ice to increase its viscosity. This viscous emulsion was not added drop by drop to a 0.1% polyvinylpyrrolidone solution in water with stirring to yield a w/o/w emulsion. The minute globules separated to form distinct microcapsules. The microcapsules were agitated for 2 hr to aid complete solvent evaporation. Finally, the microcapsules were filtered and vacuum-dried.

To determine actual vaccine loading, 10 mg of the microcapsules was crushed and dispersed in 1 ml of PBS, and DT was determined by ELISA. To each of 10 vials containing 1 ml of PBS, 10 mg of the microcapsules was added. These vials were placed at 37°C for *in vitro* release rate studies. One vial was estimated for its DT content in the dispersion medium each week by ELISA.

For the *in vitro* studies, three groups of 10 mice each [illegible] taken (3-month-old inbred strain of Balb/C mice). To [illegible] microcapsules equivalent to 3 Lf units were [illegible] implanted inside the right thigh. To the [illegible] three injections of 1 Lf of DT with calcium pho-

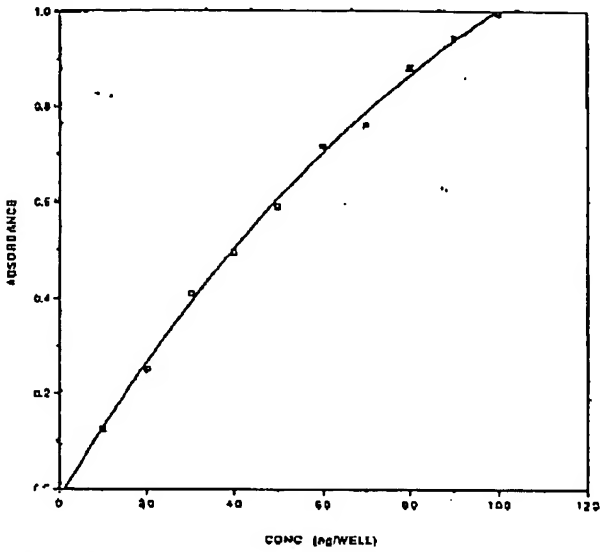

Fig. 1. Quantitative estimation of DT antigen using the developed ELISA at 492 nm. The sensitivity is 2 ng and the linearity ranges from 10 to 100 ng.

phate, each at an interval of 30 days, were given, i.e., days 0, 30, and 60. The third group served as a control.

The initial microcapsules and those retrieved after 21 days of *in vitro* degradation were subjected to scanning electron microscopic studies to determine the surface uniformity and the release characteristics. A 35 JEOL SEM instrument with 100-Å gold–palladium coating was used for this study.

RESULTS AND DISCUSSION

Poly(D,L-lactide) of 49,000 molecular weight was selected for long-term release rate studies of the toxoid. The in-water drying method gave microcapsules in the range of 30–100 μm. As this range of microcapsules can pass through

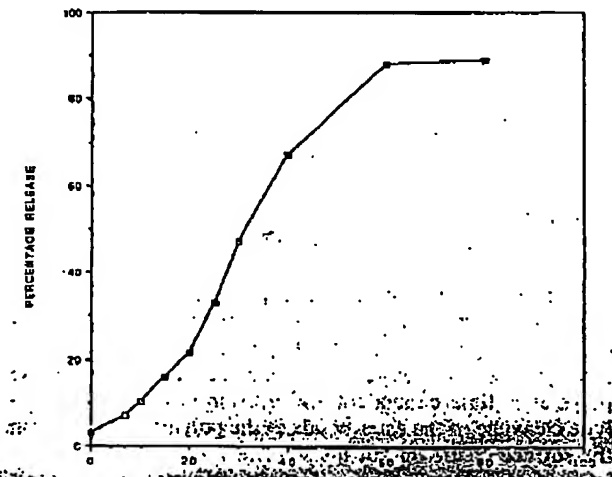

Fig. 2. *In vitro* release of the antigen from poly(D,L-lactide) microcapsules calculated as the percentage of the amount released at time [illegible] to the actual vaccine loading.

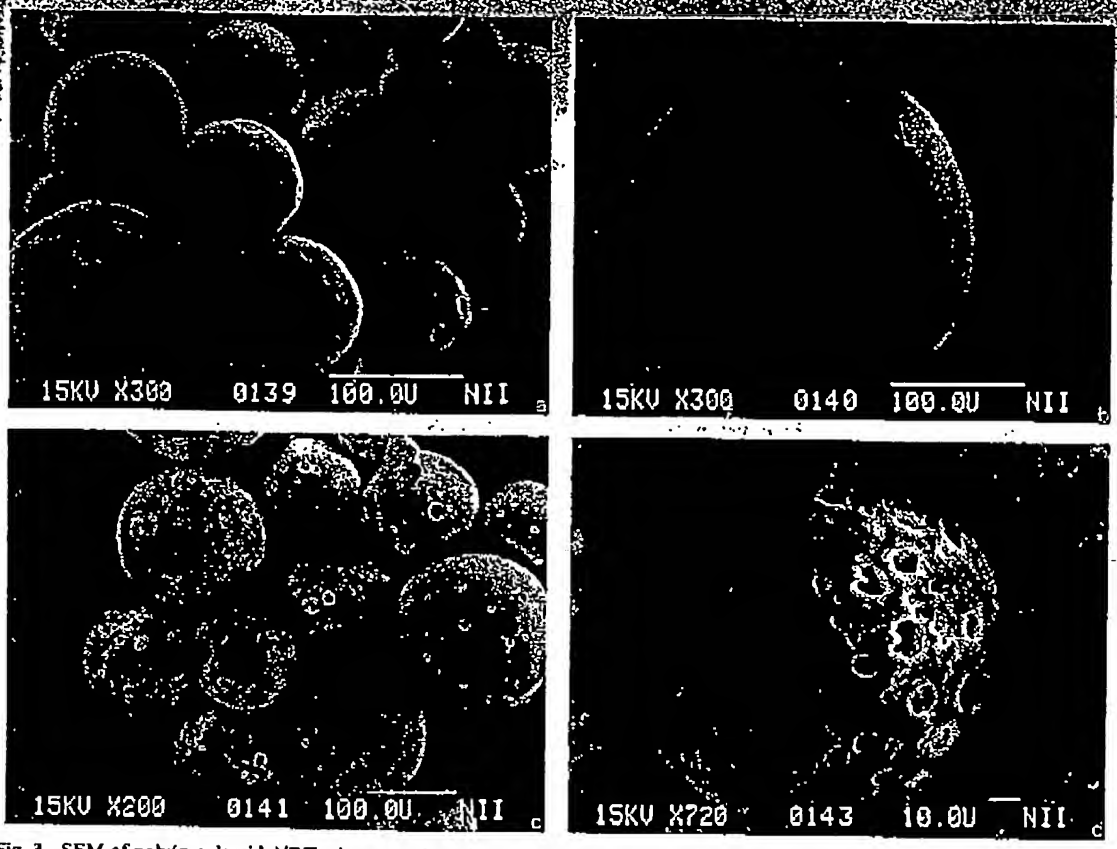

Fig. 3. SEM of poly(D,L-lactide)/DT microcapsules after complete solvent evaporation and vacuum drying. (b) SEM of a large microcapsule to exhibit surface uniformity and lack of surface porosity before initiating *in vitro* degradation. (c) SEM of poly(D,L-lactide)/DT microcapsules after 21 days of *in vitro* degradation in 50 m*M* PBS at pH 7.4. (d) SEM of poly(D,L-lactide)/DT microcapsule after 21 days of *in vitro* degradation exhibiting surface geometry and high porosity leading to greater water uptake. (a, b) ×300, (c) ×200, and (d) ×720; reduced 35% for reproduction.

an 18-gauge hypodermic needle, the preparations were not separated on basis of their size. The actual Lf units of DT in the microcapsules was determined to be 93% of theoretical. The ELISA for the detection of *in vitro* release rates of DT was highly sensitive within the range of 10–100 ng (Fig. 1) and all samples were diluted to fall within this sensitivity range.

The *in vitro* DT release from the microcapsules was 88% of the actual vaccine loading in 60 days (Fig. 2). Thus the microcapsule matrix erodes sufficiently over 60 days to allow depletion of the macromolecule through development of pores and craters on its surface, seen in the SEM studies. During the process of microencapsulation, a small loss of antigenic determinants could have occurred as the ELISA detected only 88% of the total vaccine loading. The SEM photographs (Figs. 3a–d) show that the degradation of the polymer is time dependent and erosion based. The hydrolytic cleavage of the ester bonds in the polymer backbone on the surface of the microcapsules leads to its degradation to lactic acid monomers, resulting in the formation of craters and channels through which the antigen is released. As the erosion is time dependent, a gradual but continuous release of the antigen occurs from the microcapsule.

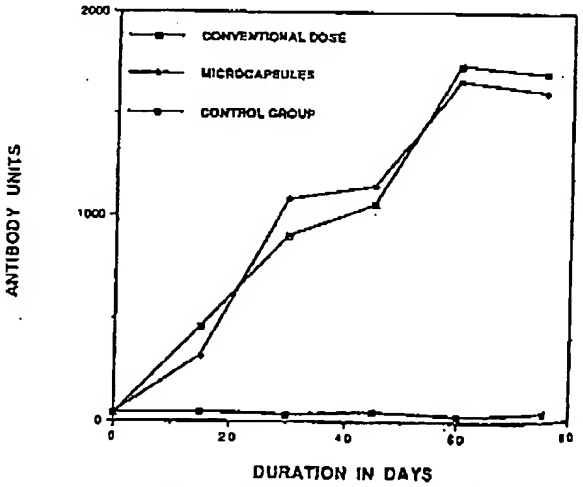

Fig. 4. *In vivo* antibody titers in the three groups of BALB/c mice. The antibody units correspond to the product of the dilution of the serum to its absorbance. (—□—) Group receiving conventional three-injection schedule; (—♦—) group receiving poly(D,L-lactide) microcapsules; (—■—) the control group.

To predict the vaccine release from theoretical considerations, the following equation can be applied, which is valid for an erosion-based release of the drug (14).

$$M_t/M_\infty = 1 - (1 - K_0 t/C_0 a)^n$$

M_t/M_∞ = fractional drug release
K_0 = device erosion constant
C_0 = initial drug concentration uniformly distributed
a = radius of sphere or half-thickness of the slab
n = shape term: 1 for a slab, 2 for a cylinder, and 3 for a sphere
t = time period

However, by determining the degradation rate of a polymer having molecular weight and knowing its initial drug concentration, it is possible to predict the release rate of the molecule occurring only through erosion. Another important parameter controlling the rate of degradation of the polymer is the pH and ionic strength of the dispersion medium (15–17). Further, as the degradation of the polymer proceeds, it becomes more hydrophilic and shows greater water intake because of the surface geometry of the degrading polymer, leading to accelerated degradation and depletion of the matrix (18).

The antibody titers till day 75 in the group immunized with the microcapsules were comparable with those in the group receiving the conventional three-injection schedule (Fig. 1). Therefore a slow antigen release yields comparable antibody titers and did not seem to develop any tolerance in the animal model. Whether a triphasic pulsatile release would offer a better result remains to be studied.

We conclude that a denatured protein preparation such as TT can be encapsulated using poly(D,L-lactide) without loss of its immunogenicity. The continuous release of the protein elicits antibody titers over 75 days that were comparable to those obtained with the conventional toxoid dose adsorbed on aluminum phosphate as an adjuvant.

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Induction of secretory immunity with bioadhesive poly (D,L-lactide-co-glycolide) microparticles containing *Streptococcus sobrinus* glucosyltransferase

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Smith DJ, Trantolo DJ, King WF, Gusek EJ, Fackler PH, Gresser JD, De Souza VL, Wise DL. Induction of secretory immunity with bioadhesive poly (D,L-lactide-co-glycolide) microparticles containing Streptococcus sobrinus *glucosyltransferase.*
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The effect of mucosal delivery of *Streptococcus sobrinus* glucosyltransferase (GTF) in bioadhesive poly (D,L-lactide-co-glycolide) (PLGA) microparticles on induction of salivary IgA and serum IgG antibody responses was measured in Sprague-Dawley rats. Preparations of GTF/PLGA/gelatin microparticles, or PLGA/gelatin microparticles or GTF in alum, were administered four times at weekly intervals by intranasal or intragastric routes. Two subcutaneous injections of GTF in PLGA/gelatin microparticles or in alum were given to separate groups of rats. Significant elevations in salivary IgA antibody levels to *S. sobrinus* GTF were observed only in the groups immunized intranasally 28 days after immunizations were begun. Five of six rats given the GTF microparticles intranasally had positive salivary IgA antibody responses to GTF, and the mean salivary IgA antibody level of this group was 30-fold higher than any other mucosally or systemically immunized group. Salivary IgA responses in the GTF-microparticle group remained significantly higher than all other mucosally immunized groups for at least 10 weeks after the primary immunization. All rats in this group demonstrated aspects of anamnesis following a more limited secondary course of intranasal administration. Intranasal administration of GTF in microparticles also induced a serum IgG response to GTF in some rats. After secondary intranasal GTF microparticle administration, several rats had sustained serum IgG antibody levels that were within the range of sera from rats subcutaneously injected with GTF in microparticles or in alum. Thus intranasal delivery of GTF-containing bioadhesive microparticles induced the highest and longest lasting salivary immune response of any mucosal or systemic route or vehicle tested and could be expected to be a useful method for induction of mucosal immunity.

Key words: PLGA; microparticles; glucosyltransferase; *Streptococcus sobrinus*; saliva; IgA

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Glucosyltransferases contribute to the molecular pathogenesis of mutans streptococci, chiefly because of their ability to synthesize extracellular glucan from sucrose (5). Strategies for immune intervention in the processes leading to dental caries, therefore, have included the development of immune responses to glucosyltransferase (GTF) (14). Glucan synthesis, which is the consequence of the catalytic action of GTF on sucrose, is an important component of the colonization/accumulation potential of mutans streptococci, and, thus the expression of the virulence of these organisms (5). Glucosyltransferases (GTF) from mutans streptococci can induce immune responses that inhibit GTF catalytic activity (18), protect rodents from experimental dental caries (13, 19), and interfere with reaccumulation of indigenous mutans streptococci in humans (15, 16). These levels of experimental protection from mutans streptococcal colonization and disease can be achieved by topical (16), oral (13, 15) or intragastric (13) application of GTF, although levels of demonstrable IgA antibody induced tend to be low and relatively short-lived.

A continuing issue in mucosal vaccine therapy is the problem of delivering the vaccine to the site of antibody induction in an efficient and undegraded state. One approach to this problem is to incorporate antigen into microcapsules for immunization. Microspheres and microcapsules made of poly(D,L-lactide-co-glycolide) (PLGA) have been used as local delivery systems (1, 2, 7) because of their ability to control the rate of release, evade pre-existent antibody clearance mechanisms and degrade slowly without eliciting an inflammatory response to the polymer. Particularization of antigen also optimizes association with M cells overlaying inductive regions of the secretory immune system (3, 11). However, the use of microcapsules as delivery vehicles for protein antigens has been somewhat problematic since most formulations require the use of organic solvents, for example methylene chloride, which can denature the antigen and render much of it biologically inactive. Limited bioadhesion and inappropriate particle size for uptake by M cells have also limited the usefulness of this approach. However, a modification in PLGA microparticle manufacture has addressed the problem of antigen degradation by incorporating antigen into microparticles in an aqueous phase (6). Increasing the bioadhesion of PLGA microparticles has also been shown to enhance particle uptake (20). These antigen-loaded microparticles, may, therefore, have the potential to induce a long lasting mucosal immune response. Thus, the present study was designed to explore the ability of microparticles containing *S. sobrinus* glucosyltransferase as antigen, and 1% gelatin as bioadhesive, to induce mucosal immunity by intranasal, intragastric or subcutaneous routes of administration.

Material and methods

Glucosyltransferases

GTF from *Streptococcus sobrinus* strain 6715 was obtained as previously described (18). After bacterial growth in glucose-containing defined medium, enzymes were isolated from culture medium by affinity chromatography on Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, NJ) with 3 M guandinine HCl as the eluting solvent. This GTF-rich pool was then subjected to FPLC liquid chromatography on Superose 6 (Pharmacia) with 6 M guanidine HCl for elution. The gel filtration step removes non-GTF and other glucan-binding proteins from the GTF preparation, as evidenced by the fact that the protein bands observed after SDS-polyacrylamide gel electrophoresis were all associated with enzymatic activity after incubation of duplicate gels in sucrose. The *Streptococcus sobrinus* GTF preparation obtained after gel filtration on Superose 6 contained a mixture of GTF isozymes including GTF-I and GTF-S but was essentially free of other proteins. Approximately 90% of the glucan synthesized by this preparation was water-insoluble, under the conditions of the assay described below. This preparation was used for microparticle preparation, immunization experiments, enzyme assays and enzyme-linked immunosorbent assay (ELISA) measurement of antibody activity.

Microparticle preparation

S. sobrinus GTF was incorporated into the PLGA-based biodegradable carrier using 1% gelatin as a bioadhesive agent. Bioadhesives employing secondary forces are most relevant for temporary attachment to M-cell surfaces prior to endocytosis, taking advantage of electrostatic interactions including hydrogen bonding between charged hydrophilic groups (carboxyl, amino, sulfate), as well as hydrogen bonding involving uncharged hydroxyl groups (4). Mechanical bonding is facilitated by these secondary forces (12). Studies with mouse ligated loop models indicated that gelatin had superior bioadhesive properties compared with Eudragit and lectin; thus gelatin was selected for use.

The PLGA copolymer (Boehringer Ingelheim Chemicals, Mannheim, Germany) was characterized with respect to molecular weight distribution using gel permeation chromatography (Ultrastyragel 100 Å and Ultrastyragel Linear columns). A ratio of 75:25 lactide: glycolide was used in this experiment. A low density polymer foam was prepared by lyophilization of a polymer solution in glacial acetic acid. The polymer foam was cryogenically ground in a Tekmar Model A-10 analytical mill (20,000 rpm) equipped with a cryogenic well, cooled with liquid nitrogen, enabling low temperature particle size reduction.

S. sobrinus GTF (10%) and gelatin (1%) were dry mixed (w/w) with PLGA 75:25 polymer. The matrix was then compressed and extruded at a pressure of 15,000–20,000 psi and at a temperature of 45–55°C. High-pressure extrusions ensure that the protein is fully incorporated with the polymer lattice with a concomitant reduction in particle porosity: this minimizes premature release of the active agent. The sized PLGA/GTF/gelatin blend was loaded into a mold and extruded through a 2.4-mm-diameter die using a Compac Type MPC 40-1 hydraulic press. Following extrusion the matrix was again cryogenically ground in a Tekmar mill and sieved to retain particles less than 45 µm. Gelatin/PLGA microparticles that did not contain GTF were prepared under similar conditions for use as a control antigen preparation.

Immunization protocol

The mucosal immunogenicity of GTF/1% gelatin dry mixed with PLGA-75:25, compressed and Tekmar ground microparticles was measured in six groups (groups A through F) of Sprague-Dawley rats, using the protocol shown in Table 1. Prior to immunization, all rats were bled from the tail vein and salivated for 10 min by gravity collection (10 mg pilocarpine nitrate/kilogram rat weight) under ether anaesthesia. Immunization was initiated

Table 1. Immunization protocol

Groups	*n*	Route	GTF (μg)	Formulation	Protocol: Primary immunization days	Protocol: Secondary immunization days
A	6	intranasal	0	1% gelatin/PLGA	0, 7, 14, 21	103, 110, 117
B	6	intranasal	60	GTF/1% gelatin/PLGA	0, 7, 14, 21	103, 110, 117
C	5	intranasal	20	soluble GTF+alum	0, 7, 14, 21	103, 110, 117
D	6	intragastric	60	GTF/1% gelatin/PLGA	0, 7, 14, 21	103, 110, 117
E	4	subcutaneous	60	GTF/1% gelatin/PLGA	0	28
F	4	subcutaneous	5	soluble GTF+alum	0	28

when rats were approximately 45 days old. Four weekly primary mucosal immunizations and three weekly secondary mucosal immunizations were performed in groups A–D. Subcutaneously injected rats (groups E and F) were immunized in the vicinity of the salivary glands on days 0 and 28. Rats that were mucosally immunized with antigen in PLGA were given a three-fold higher dose than rats immunized intranasally with GTF in soluble form because the release data (Table 2) indicated that somewhat less than one third of the enzyme in the GTF-loaded microparticles was released *in vitro*. Animals injected with GTF/gelatin/PLGA were given the same GTF dose as those mucosally immunized with the gelatin-PLGA microparticles. All rats were bled and salivated on 1, 3, 7 and 11 weeks after primary immunization and 1, 3 and 7 weeks after secondary immunization. Serum IgG and salivary IgA antibody were then measured by ELISA.

Rats in groups A–C were intranasally immunized with 0.03 ml of microparticle mixture distributed equally between both nostrils with an Ependorf pipet. This dose was well tolerated by the intranasally immunized animals; thus, no anesthesia was required for antigen administration. Rats in group D were immunized intragastrically with 0.03 ml microparticle mixture via a 20 gauge, 1.5-inch intubation needle (Pooler & Sons, New Hyde Park, NY). Food was withdrawn 4–6 h before immunization and 0.1 ml of 0.2 M sodium bicarbonate was added immediately prior to antigen administration to reduce stomach acidity. Rats in groups E and F were subcutaneously injected with 60 μg GTF/PLGA or 5 μg GTF in aluminum phosphate.

ELISA

Serum IgG and salivary IgA antibodies were tested by enzyme-linked immunosorbent assay (ELISA). Polystyrene microtiter plates (Flow Laboratories, San Francisco, CA) were coated with 0.2 ml of 0.5 μg/ml of *S. sobrinus* GTF. Antibody activity was then measured by incubation with 1:400 and 1:10,000-fold dilutions of sera, or 1:4, 1:16 or 1:64-fold dilution of saliva. Plates were then developed for IgG antibody with rabbit anti-rat IgG, followed in sequence by alkaline phosphatase goat anti-rabbit IgG (Biosource, Camarillo, CA) and *p*-nitrophenylphosphate (Sigma Chemical Co., St. Louis, MO). A mouse monoclonal reagent to rat α chain (Zymed, South San Francisco, CA) was used with biotinylated goat anti-mouse IgG (Zymed) and avidin-alkaline phosphatase (Cappel, West Chester, PA) to reveal levels of salivary IgA antibody to peptides. Reactivity was recorded as absorbance ($A_{405\ nm}$) in a micro plate reader (Biotek Instruments, Winooski, VT). Data are reported as ELISA units (EU) which were calculated relative to the reactivity of appropriate reference sera.

Measurement of *S. sobrinus* GTF enzyme activity

Nine milligrams of microparticles, initially loaded with 0.9 mg of GTF, were incubated at 37°C on a platform rocker in polypropylene tubes containing 1.6 ml of phosphate buffered saline, pH 6.5, with 0.2% bovine serum albumin and 0.2% sodium azide. At each experimental time point aliquots were removed, spun in a microcentrifuge, and the supernatants tested for released GTF. The release of GTF from microparticles was assayed by measuring the ability of the *S. sobrinus* GTF to catalyze the synthesis of glucan from sucrose. In the assay, 1.7 mg sucrose and 9 nCi of [^{14}C-glucose]-sucrose (approximately 17,000 cpm) are added to 0.1 ml of phosphate-buffered saline, pH 6.8 with added 0.02% sodium azide, 0.1% bovine serum albumin, and 0.3 mg dextran primer. Incubation proceeded for 5 h at 37°C, after which glucan was precipitated in 3 volumes of 95% ethanol and radioactivity determined as previously described (18). Assays were performed in duplicate.

Table 2. In vitro release of GTF from PLGA microparticles

Time of sample incubation at 37°C (h)	1% gelatin/PLGA (cpm ^{14}C-glucan)[a]	GTF/1% gelatin/PLGA (cpm ^{14}C-glucan)[a]	GTF activity released/ total GTF activity added to microparticle
0.2		870	0.13
2	37	1740	0.26
20		1587	0.24
52	38	1226	0.18
96		1012	0.15

[a] Release of GTF activity from microparticles was measured by the incorporation of ^{14}C glucose from ^{14}C-sucrose, as described in Material and methods.

Results

In vitro release of GTF from PLGA/1% gelatin microparticles

The release of GTF from the microparticles after incubation at 37°C in PBS-azide was measured by the ability of released GTF to incorporate ^{14}C glucose from labeled sucrose into glucan, as described in Material and methods. The activity of an amount of free GTF, equivalent to fully loaded microparticles and incubated under the same

conditions as the GTF PLGA microparticles, was also measured and used to determine the percentage of enzyme released. The results are shown in Table 2. The maximum release of active GTF from the microparticles was found to occur after 2 h of incubation under the conditions indicated. The amount released at this time corresponded to 26% of the GTF activity calculated to be incorporated into the equivalent amount of microparticles. This release rate was somewhat more rapid than that seen for microparticles containing a prototype α-amylase protein (39% release of enzyme activity at 19 h of incubation), but was considered to be desirable for mucosal immunization given the potentially limited period of exposure of the microparticles to the nasal or intestinal surfaces.

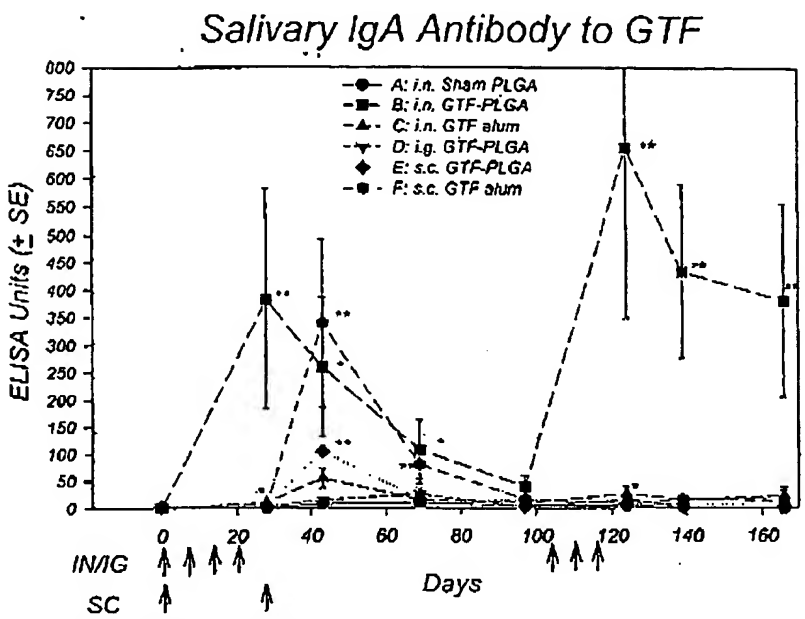

Fig. 1. Mean and standard deviations of salivary IgA antibody levels to *S. sobrinus* GTF for all groups throughout the full course of the experiment. Group designations and treatment are indicated in the legend. Intranasal/intragastric (IN/IG) or subcutaneous (SC) immunization events are indicated by arrows beneath the abscissa. The levels of statistical significance, compared with the sham-immunized group A are indicated by asterisks (* $P<0.05$; ** $P<0.01$: Kruskal-Wallis ANOVA on ranks).

Salivary IgA antibody responses to GTF

The mucosal immune responses of animals immunized intranasally, intragastrically or subcutaneously with GTF or placebo in gelatin-coated microparticles or alum are shown in Fig. 1. Significant elevations in salivary IgA antibody levels to *S. sobrinus* GTF were observed only in the groups immunized intranasally on day 28. Five of six rats given the GTF/gelatin/PLGA microparticles intranasally had elevated salivary IgA antibody to GTF. Two of these animals had antibody levels which were detectable at salivary dilution of at least 1:128. Two of the five rats given GTF intranasally in alum had low but positive responses. However, the mean response of the alum group was less than 1/10 that of the PLGA groups at this time. The GTF/gelatin/PLGA intranasally administered group B antibody levels remained significantly elevated above the sham group A levels for at least 69 days of the primary response. Three months after initial intranasal immunization, salivary IgA antibody levels continued to be high in two of the six group B rats (74 ELISA units and 119 ELISA units), although the mean group B antibody level was not significantly greater than group A. In contrast to group B animals, the rats in groups E and F, who received a single subcutaneous injection of GTF in either microparticles or microparticles, did not show a salivary IgA antibody response until day 42, following a second injection on day 28.

Following a more limited secondary mucosal exposure to antigen on days 103, 110 and 117 (Fig. 1), all rats intranasally immunized with GTF/gelatin/PLGA microparticles demonstrated a salivary IgA antibody response to GTF. Seven days after completion of the secondary immunization regime (day 124), the mean salivary IgA antibody response in this group was nearly twice that detected on day 28. Furthermore, mean salivary IgA antibody levels during the secondary phase of the response to intranasally administered GTF/gelatin/PLGA microparticles remained at or above peak primary antibody levels for at least 42 days. A low but significant increase in salivary IgA antibody was observed only on day 124 in the group C rats who had been given GTF in alum intranasally. No significant increase was observed in the intragastrically immunized group D. The salivary IgA antibody levels of groups E and F increased to significant levels after a second injection of GTF microparticles or GTF in alum on day 28. However, neither injected group achieved the level or duration of secretion of salivary IgA antibody seen after the second immunization regime given to the intranasally immunized group B.

Serum IgG antibody responses to GTF

Serum IgG antibody responses to GTF were also measured in all animals prior to immunization and throughout the course of the experiment (Fig. 2). Serum IgG antibody to GTF could be detected in all subcutaneously injected animals of either group E or F and in four of the six intranasally immunized rats of group B, 4 weeks after initial exposure to the antigen. No other group had significantly elevated serum IgG antibody to GTF at this time, although serum IgG antibody could be detected in at least two group C rats.

After a second injection of GTF in alum or microparticles, serum IgG antibody levels increased more than tenfold and remained high for the duration of the experiment (Fig. 2). The mean serum IgG response in the group B rats remained significantly higher than all other mucosally immunized groups throughout the primary immunization period and increased significantly from the peak primary level (day 28) after a second mucosal immunization regime. Serum IgG and salivary IgA antibody levels in the group B rats showed significant positive correlations (Spearman

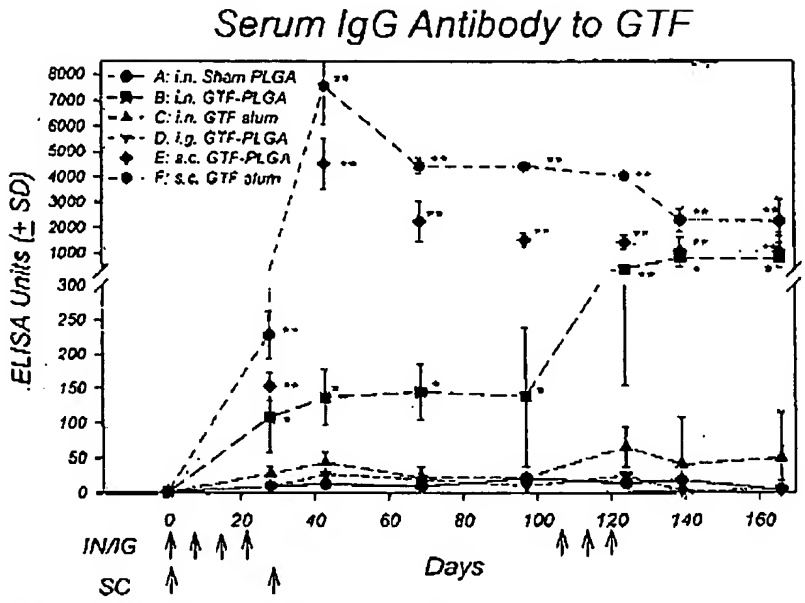

Fig. 2. Mean and standard deviations of serum IgG antibody levels to *S. sobrinus* GTF for all groups throughout the full course of the experiment. Group designations and treatments are indicated in the legend. Intranasal/intragastric (IN/IG) or subcutaneous (SC) immunization events are indicated by arrows beneath the abscissa. The levels of statistical significance, compared with the sham-immunized group A are indicated by asterisks (* $P<0.05$; ** $P<0.01$; Kruskal-Wallis ANOVA on ranks).

rank order correlation) after both primary ($r^2=0.996$) and secondary ($r^2=1.00$) immunization.

Discussion

The results reported herein support the hypothesis that the dry mix method of incorporation of *S. sobrinus* GTF into PLGA microparticles, followed by compression, extrusion and grinding do not radically affect the structure of this enzyme. This conclusion is based on the fact that significant enzymatic activity (26%) could be detected in the supernatant of GTF-PLGA microparticles incubated for 2 or more hours under the conditions described. This observation was supported by preliminary release experiments with α-amylase. Amylases are a group of enzymes that share with mutans streptococcal GTFs the ability to release glucose from polysaccharides. Recent modeling studies indicate that the catalytic domain of GTF bears striking homology with α-amylases with regard to secondary domain structure and with regard to the essential residues involved in catalytic activity (8). Thus α-amylase provided a useful prototype to measure the release rate and effect of particle preparation on enzymatic activity. Up to 39% of the loaded α-amylase activity could be recovered after 19 h of incubation at 37°C in phosphate-buffered saline with azide (data not shown). The slightly higher amount of α-amylase activity compared with GTF activity released might be explained by the larger size of GTF. However, both sets of experiments indicate that the microparticle preparation method preserves much if not all of the native structure of these two proteins, based on the preservation of their enzyme activity. Thus, immunization with GTF microparticles could be expected to include immune responses to some conformationally dependent epitopes.

Based on the amount of release of enzymatically active GTF, intranasally immunized PLGA-GTF rats were given a three-fold greater dose (calculated from the amount of GTF initially added in the manufacturing process) than rats intranasally immunized with soluble GTF. It is conceivable that non-enzymatically active, but immunogenic GTF or fragments thereof could also be released, thereby increasing the actual dose. We have qualitative evidence from previous experiments that some GTF protein remains in the microparticles for extended periods. Thus not all of the protein is released. Furthermore, if the GTF release data for the PLGA-GTF microparticles (26%) represents all the immunogenic GTF released, then the PLGA-GTF microparticle rats would have received only 78% of the dose received by the rats immunized intranasally with soluble GTF. These observations, taken together with the finding that the subcutaneously injected PLGA-GTF rats consistently demonstrated significantly lower salivary and serum responses than did the subcutaneously injected soluble GTF rats, despite the fact that they received a three-fold higher GTF dose (Table 1), supports the notion that the intranasally immunized PLGA-GTF rats did not receive a higher useful GTF dose than their soluble GTF immunized counterparts.

Intranasal administration of antigen has been shown to induce salivary IgA antibody (17) at levels that are generally superior to those induced intragastrically (21). In the present study, intranasal application of GTF-PLGA microparticles was far superior to other routes (intragastric or subcutaneous) or formulation (alum) for induction of salivary IgA immunity after primary or secondary immunization regimens. The superiority of the intranasal, versus the intragastric route for induction of salivary antibody may be a consequence of the relatively higher dilution of particulate antigen in the intestine or the greater potential for rapid breakdown of antigen in the gut versus nasal/tonsillar area. A degree of compartmentalization of the mucosal immune response may also account for this difference, in that nasal/tonsillar induction of mucosal immunity may favor a greater expression of IgA antibody in the saliva than would induction of gut-associated lymphatic tissue, which favors a predominantly intestinal expression of antibody activity (9).

Microparticle bioadhesiveness may increase the dose of antigen available to induce local immune responses by increasing the time that antigen-laden biodegradable particles are in contact with mucosal surfaces. Montgomery & Rafferty (10) have shown that oral administration of bioadhesive degradable starch microparticles containing dinitrophenyl-bovine serum albumen, in combination with L-α-lysophosphatidylcholine as a penetration enhancer, po-

tentiated long-lived salivary IgA responses, compared with antigen delivered in soluble form. In the present study both GTF-loaded and control PLGA preparations given intranasally or intragastrically contained gelatin to increase the bioadhesive properties of the microparticles. Although the present experiments do not clarify the extent to which the use of gelatin improved the immune response, previous work with the mouse ligated intestinal loop model (3) indicated that incorporation of gelatin enhanced microparticle uptake into phagocytic cells within the follicle associated epithelium.

Intranasally administered GTF-PLGA microparticles induced a salivary IgA antibody response to GTF that was relatively long lasting compared with other routes and modes, especially after secondary stimulation (Fig. 1). This mucosal IgA antibody response had aspects of anamnesis in that the peak secondary response was higher than the peak primary response (604 ELISA units versus 383 ELISA units). Furthermore, salivary IgA antibody was detected at a higher level for a longer period of time in the secondary, compared with the primary, phase of the response to the intranasally applied GTF-PLGA microparticles. Another important feature was that the secondary exposure to the intranasal GTF-PLGA microparticles converted all the responses of rats in whom either no or low primary salivary IgA antibody responses had been detected. This suggests that repeated intranasal application of appropriate antigen in these bioadhesive microparticles could be expected to induce an immune response in most subjects, despite the typically wide range in secretory immune responses seen to indigenous and artificial antigens.

A significant serum IgG antibody response was also seen in several rats that were intranasally immunized with GTF-PLGA microparticles (Fig. 2). This response was highly associated with the degree of salivary IgA response among animals of this group. The serum IgG responses seen in the most responsive group B rats were within the range of those seen in the systemically immunized rats by the end of the experiment (day 169: Fig. 2). The precise mechanisms for these responses are as yet unclear. One explanation would be that in the most responsive group B rats, some GTF-PLGA microparticle fragments were phagocytized and carried to the cervical lymph nodes, where they continued to supply antigen-presenting cells with GTF for induction of systemic immunity. In this regard, Wu & Russell (21) have reported that antibody-secreting cells appeared in both the superficial and posterior cervical lymph nodes shortly after completion of intranasal administration of soluble *Streptococcus mutans* Ag I/II conjugated to cholera toxin B subunit.

The enhancement of the expression of immunity within the oral cavity after delivery of GTF in bioadhesive microparticles indicates that this immunization regime may be particularly efficient in the induction of antibody that could interfere with the colonization and accumulation of cariogenic mucosal streptococci in the oral cavity. Protection could be expected not only from the enhancement of salivary immunity but also from systemic IgG antibody that enters the oral cavity via the gingival crevicular fluid route. Intranasal application of similar formulations of bioadhesive microparticles, loaded with appropriate antigen, also holds promise for the enhancement of immunity to a variety of upper respiratory and oral infections.

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PLGA drug delivery

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Tetanus toxoid and synthetic malaria antigen containing poly(lactide)/poly(lactide-co-glycolide) microspheres: importance of polymer degradation and antigen release for immune response

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Abstract

The importance of in vitro degradation of poly(lactide)/poly(lactide-co-glycolide) (PLA/PLGA) microspheres and of the concomitant in vitro release of a natural and a synthetic antigen for eliciting immune response was studied in mice. A variety of PLAs and PLGAs differing in molecular weight ($\bar{M}_w$ of 14–130 kDa) and in polymer composition (lactic/glycolic acid ratio of 100:00, 75:25, and 50:50) were examined for their in vitro degradation, which ranged from approximately 4 to 20 weeks. Three specific polymers were then selected for microencapsulation of the two antigens tetanus toxoid (TT) and a weakly immunogenic synthetic branched malaria peptide (P30B2). The in vitro release data showed that antigen delivery correlates fairly well with polymer degradation giving rise to a distinct burst release during the first 24 h and an additional release pulse towards the end of polymer degradation. After single subcutaneous administration in mice, long lasting high antibody titers were obtained with the antigen containing microspheres, as compared to TT adsorbed on alum or to P30B2 in Incomplete Freund's Adjuvant. Moreover, the immune responses induced by microspheres were clearly influenced by the antigen release kinetics, the polymer type and the size of the microspheres. The results demonstrate the immunopotentiating properties of the biodegradable microspheres and their potential to elicit long-lasting immune responses after single administration when tailoring in vitro release characteristics and particle size.

Keywords: Poly(lactide) (PLA); Poly(lactide-co-glycolide) (PLGA); Biodegradable microspheres; Polymer degradation; Tetanus toxoid; Synthetic malaria antigen; Antigen release; Immune responses

1. Introduction

The search for new immunological adjuvants and antigen delivery systems has attracted the interest of various national and international health institutes and organizations, as well as of academic and industrial research scientists. Three major objectives of practical importance have been defined to improve existing vaccines. Clearly, formulations or delivery systems should (i) mimic booster doses after single parenteral administration, (ii) exhibit adequate immunostimulating properties for weakly immunogenic (synthetic) antigens, and (iii) elicit a strong immune response after nasal or oral administration. In this light, biodegradable microspheres (MS) based on poly(lactide) (PLA) or poly(lactide-co-glycolide) (PLGA) are probably the most promising of all the

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antigen delivery systems studied so far. In addition to the immunostimulating properties of most other adjuvant formulations or delivery systems, biodegradable MS also provide prolonged and even pulsatile antigen release over several months, thereby mimicking conventional immunization schedules [1–4]. It is the particular goal of replacing the necessary booster injections of conventional vaccines by a single injection of antigen containing microspheres which has been addressed by the WHO in an ongoing program initiated in 1989 [5,6]. In addition, the demonstrated targeting of biodegradable microspheres to the Peyer's patches of the GI-tract or to the nasal mucosa holds great promise for peroral and nasal immunization [7–10].

The basis for the present interest in PLA/PLGA-based biodegradable microspheres for vaccine delivery was laid undoubtedly in the late 1970s to mid-1980s, particularly by pioneering investigations on (i) single-step immunization by sustained antigen release [11,12], (ii) peptide drugs being released from PLGA-microspheres in a pulsatile manner [13], and on (iii) the biodegradation of and tissue reaction to PLA/PLGA-microspheres [14,15].

Various studies have been conducted to clarify the in vitro and in vivo degradation mechanisms of PLA/PLGA polymers as it became evident that drug release kinetics and pharmacological responses greatly depend on the polymer characteristics and biodegradation behaviour [16–22]. These investigations revealed that in particular the release of macromolecules is closely related to polymer degradation and, hence, to copolymer composition and molecular weight. Moreover, it has been found that the degradation pattern of polymers obtained from different suppliers is also affected by polymer quality, i.e. by the presence of low molecular weight fractions and monomers [23]. Special attention has been paid further to the role of water involved in polymer degradation and its influence on glass transition temperature, T_g [24,25]. On the other side, relatively little knowledge is available at present on the interaction between polymer and protein under release conditions. It has been reported that osmotic effects and ionic interactions between polymer end groups and the amide bond or free amino groups of polypeptides greatly affect the release pattern of proteinaceous compounds from microspheres [26–28]. It has also been recognized for some time, and reemphasized more recently, that during polymer degradation, the hydrolysis products are likely to create an acidic environment inside the microspheres and within their microenvironment, which may severely compromise protein stability in in vitro release studies and also in an in vivo situation [16,28,29]. However, general conclusions about the importance of an acidic microenvironment on protein stability cannot been drawn as every protein forms its own case.

Over the past few years, the particular aspect of antigen delivery from biodegradable MS and its relevance for the immune response has been investigated by several groups. Most of these studies have focused on the natural antigens tetanus toxoid (TT) [1,2,10,30] and diphtheria toxoid (DT) [31], and only very few have considered synthetic antigens [3]. Generally, some correlations have been found between the kinetics of polymer degradation and of antigen release, but not between in vitro release kinetics and immune response. All of these studies confirm the very pronounced immunostimulating properties of microspheres.

Here, we report on the correlation between polymer degradation and antigen release from microspheres, and on the importance of their release kinetics and particle size for immune response. Investigations on two different types of antigens are being summarized, i.e. conventional tetanus toxoid and a synthetic antimalarial antigen.

2. Experimental

2.1. Materials

Lyophilized tetanus toxoid (TT) (approximately 90 Lf/mg protein, Institute Merieux, Lyon, France) was provided by WHO (Geneva, Switzerland). A tetra-branched synthetic peptide composed of an universal T helper epitope from tetanus toxin (947–967) and a B-cell epitope from the repetitive region of *Plasmodium berghei* circumsporozoite protein (P30B2) was synthesized by using the F-moc strategy [32]. The molecular weights of the two antigens are approximately 150 000 for TT and 16 000 for P30B2. Various poly(D,L-lactides) (PLA)

and poly(D,L-lactide-co-glycolides) (PLGA) differing in molecular weight and composition were purchased from Boehringer Ingelheim, Ingelheim, Germany; they included the PLAs Resomer® R202 ($\bar{M}_w$ 14 600), R203 ($\bar{M}_w$ 23 300) and R206 ($\bar{M}_w$ 129 700), the PLGAs 50:50 Resomer® RG502 ($\bar{M}_w$ 13 700) and RG503 ($\bar{M}_w$ 35 100), and the PLGAs 75:25 Resomer® RG752 ($\bar{M}_w$ 17 000) and RG755 ($\bar{M}_w$ 68 600). Hereafter, the following designations will be used to specify the type of polymer: PLA-14 kDa, PLA-23 kDa, PLA-130 kDa, PLGA50:50-14 kDa, PLGA50:50–35 kDa, PLGA75:25-17 kDa and PLGA75:25–69 kDa. All other chemicals used were of analytical grade (from Fluka, Buchs, Switzerland), unless otherwise specified.

2.2. Preparation of microspheres

Empty and antigen loaded microspheres (MS) were prepared by the two microencapsulation techniques, spray-drying (SD) and coacervation (CO), as described in detail elsewhere [33,34]. Briefly, in the spray-drying process, 100 mg of TT or 20 mg of P30B2 were dissolved in 2.0 ml water. The resulting antigen solutions were finely dispersed in 100.0 g (for TT) or 40.0 g (for P30B2) of a 5% (w/w) polymer solution in ethyl formate by means of an ultrasonic processor. The W/O-dispersions were subsequently spray-dried in a laboratory spray-dryer (Model 190, Büchi, Flawil, Switzerland). The microspheres were washed with 0.1% (w/w) Synperonic® F68 solution (ICI, Middlesbrough, UK), collected on a 0.2 μm cellulose acetate membrane filter and dried under vacuum for 24 h.

In the coacervation process, the polymers were dissolved in dichloromethane (DCM) at a concentration of 10% (w/w), wherein the antigen solutions were dispersed, as described above. The dispersion was introduced into a jacketed vessel (250 ml) equipped with baffles and an anchor stirrer. Coacervation was induced by introducing a predetermined necessary amount of silicone oil (DC-200, 1070 mPa/s); with respect to the total mass of the coacervation mixture, the percentage of added silicone oil was 73% for PLA-14 kDa, 70% for PLA-23 kDa, 55% for PLA-130 kDa, 68% for PLGA75:25-17 kDa, 53% for PLGA75:25–69 kDa, 58% for PLGA50:50-14 kDa and 47% for PLGA50:50-35 kDa. Stirring was set at 1000 rev./min and the temperature maintained at 10°C. The stable coacervation dispersion was slowly poured into 1200 ml of hardening agent (octamethylcyclotetrasiloxane, OMCTS) to solidify the microspheres. Stirring was continued for 30 min, and the microspheres collected on a sintered glass filter and washed with 100 ml hexane. The product was then air-dried for 5 min and resuspended three times in 0.1% (w/w) Synperonic® F68 solution. Drying took place under the conditions specified above.

2.3. Polymer molecular weight and in vitro polymer degradation

The polymers used were characterized by gel permeation chromatography (GPC) for molecular weights, $\bar{M}_w$ and $\bar{M}_n$, and polydispersity, P. The polymers were dissolved at a concentration of 0.2% (w/w) in tetrahydrofuran (THF) (purity > 99.5%, stabilized with butylhydroxytoluene, residual water < 0.01%; Scharlau, ETG-Chemie, Tägerig, Switzerland). Toluene was used as internal standard at a concentration of 0.02% (w/w). A 100-μl aliquot of the polymer solution was injected into a 20 μl loop (Model 7125, Rheodyne, Berkley, USA) of a HPLC-system (Pump L 6000, Merck Hitachi, Merck ABS, Dietikon, Switzerland) and separated on a mixed gel column with a molecular weight separation range of 0.2 to 2000 (PL-gel 5 μm, 300 × 7.5 mm^2, Polymer Laboratories, Shropshire, UK). Column calibration was performed with a monodisperse polystyrene kit covering a molecular weight range of 1.32 to 1030 (PL polystyrene-medium molecular weight kit, Polymer Laboratories). THF was used as eluent at a flow rate of 1 ml/min, and the column temperature maintained at 30°C (Column oven, Merck Hitachi). Elution profiles were detected refractometrically (RI-detector, ERC-7512, Erma, Tokyo, Japan) at 30°C and a sensitivity of 4. The data were analyzed by a GPC integrator (D-2520, Merck Hitachi).

The in vitro degradation behavior of unloaded microspheres, prepared by coacervation, was characterized by suspending 50 mg of microspheres in borosilicate vials (Chromacol®, Welwyn Garden City, UK) containing 4.0 ml of PBS pH 7.4, pre-

served with 0.02% (w/w) sodium azide. The dispersions were ultrasonicated to facilitate wetting, and the vials fixed horizontally in a drum rotating at 3 rev./min and 37°C (Drum Roller, TC 1000/3, Brouwer, Lucerne, Switzerland). At defined intervals, the samples were analyzed in triplicate. The content of each vial was centrifuged, the supernatant withdrawn, and its pH measured. The medium of the remaining samples was replaced by fresh buffer to readjust the pH. The isolated microspheres or polymeric mass was dried under vacuum for 2 h and dissolved in THF (0.2%, w/w). The filtered solutions were finally analyzed by GPC.

2.4. Morphology and size of microspheres

For morphological examination, the microspheres were mounted on double-faced adhesive tape, sputtered with platinum and viewed in a Hitachi S-700 scanning electron microscope.

Size and size distribution of microspheres were measured by laser light scattering (Mastersizer X, Malvern, Malvern, UK). Typically, 50–100 mg of microspheres were carefully pounded in a mortar with a few drops of polysorbate 20, followed by the addition of 3 ml water. The particle dispersion was transferred into a small volume presentation unit (Malvern) and ultrasonicated for 30 s at output 40 (Vibra cell, VC50T, Sonics and Materials, Danburry, USA). Calculation of particle sizes from the scattered light was based on Mie's theory accounting for the optical properties of the polymers. The refractive index of PLA and PLGA was calculated from group contributions [35].

2.5. Antigen loading in the microspheres

The antigen content was determined with 20–30 mg of antigen loaded microspheres dissolved in 2 ml dichloromethane; the dispersion was vacuum-filtered on a 0.2 μm regenerated cellulose RC58 membrane filter (Schleicher and Schuell, Dassel, Germany). Filters were washed three times with the same solvent and air dried. The antigens were eluted three times with 3 ml PBS, pH 7.4. The solution was analyzed by Bradford's protein assay (for TT) (Bio-Rad, Munich Germany) or fluorometrically (for P30B2) (Fluoromax®, Spex Industries, Edison, NJ, USA), with excitation and emission wavelengths of 278 nm and 340 nm, respectively.

2.6. In vitro antigen release

Antigen release was determined by suspending 20–100 mg of microspheres in borosilicate vials with screw caps (Chromacol®, Welwyn Garden City, UK) containing 4.0 ml of PBS pH 7.4, preserved with 0.02% sodium azide. The dispersions were ultrasonicated to facilitate wetting, and the vials fixed horizontally in a drum rotating at 3 rev./min at 37°C. At regular intervals, the supernatant from each vial was collected by centrifugation and assayed immediately by Bradford's assay (for TT) or fluorometrically, as specified above. At every time point, 3 ml of the release medium were replaced by fresh medium and the pH readjusted to 7.4.

2.7. Immunogenicity of microencapsulated antigens in mice

Antibody titers of microencapsulated antigens as compared to TT adsorbates on aluminum hydroxide (Alum) or P30B2 in Incomplete Freund's Adjuvant (IFA) were evaluated as described elsewhere [36]. In short, groups of eight BALB/c female mice 8–10 weeks of age (OLAC, Bicester Oxon, UK) received a total dose of either 20 μg (1.7 Lf) of TT or 30 μg of P30B2 in various formulations by subcutaneous injection at the base of the tail. For immunization with tetanus toxoid, five groups of mice received a single injection of TT-MS preparations (SD502, SD752, CO502, CO752 and CO206), and another group received a single injection of TT-alum (Alum-1). Correspondingly, for the immunization with P30B2, five groups of mice received a single injection of P30B2-MS (SD502, SD752, CO502, CO752 and CO206), and another group a single injection of P30B2-IFA (IFA-1). For injection, the microspheres were suspended in 100 μl of 5% (w/w) sterile lecithin solution (Ovothin® 170, Lucas Meyer, Hamburg, Germany). Lecithin was used for dispersing microspheres because of its excellent wetting and suspending properties and for its high biocompatibility and biodegradability. No effect on antibody production was observed when mice were injected with lecithin alone. After immunization,

mouse sera were collected at an interval of 2–4 weeks by tail bleeding. Specific serum antibodies were measured by ELISA, as described elsewhere [36]. The antibody titer was expressed as the reciprocal of the highest positive serum dilution. Student's *t*-test was used on logarithm-transformed data to compare antibody levels obtained from each group at various time points, according to accepted convention [44].

3. Results

Most important characteristics of a vaccine delivery system based on biodegradable polymers encompass the polymer degradation time, the microspheres' size, the antigen release kinetics and the immunological responses. All of these four parameters were investigated in this study and reported below.

3.1. In vitro polymer degradation

For selection of appropriate polymers for antigen delivery, microspheres were prepared from a series of homo- and copolymers of lactic and glycolic acid, varying in composition and molecular weight, and were subjected to an in vitro degradation study. The microspheres were prepared by coacervation and showed a monomodal size distribution in the range of 10 to 100 μm.

First, the effect of polymer composition was examined with three low molecular weight polymers (Fig. 1). Each point represents the mean of three individual samples of microspheres. The degradation profiles appear biphasic with an initial lag time, where only minor changes in molecular weight are measured, and a terminal phase characterized by an accelerated polymer degradation. The three polymer type microspheres, i.e. PLGA50:50, PLGA75:25 and PLA, differ greatly in both the lag time and in the rate of terminal degradation. In the terminal phase an exponential decrease in molecular weight, as indicated by the linear alignment of the last three or four data points of each profile in the semilogarithmic plot is clearly shown. PLGA50:50 undergoes the fastest hydrolysis with a decrease in weight average molecular weight below 1000 within 35 days. Incidentally, as oligomers with a molecular weight below 1000 are water soluble, a $\bar{M}_w$-value of about 1000 or less is considered here as 'complete' polymer degradation. Furthermore, polymer degradation was characterized by weight average $\bar{M}_w$ rather than by the number average molecular weight. $\bar{M}_w$ was preferred because tailing in the polymer peak due to low molecular weight fragments renders setting of proper integration marks difficult and, hence, would affect greatly the calculation of number average molecular weight [37]. PLGA75:25 microspheres showed a lag time of 28 days, with only a 30% decrease in $\bar{M}_w$, followed by the exponential phase between days 28 and 56. After 8 weeks, $\bar{M}_w$ was below 10% of the initial $\bar{M}_w$. Finally, PLA microspheres required more than 3 months for complete erosion. This degradation profile was characterized by a lag time of 2 months, followed by accelerated polymer degradation up to day 100. Therefore, with increasing hydrophobicity of the polymers, the initial lag times became longer and the terminal exponential degradation rate lower.

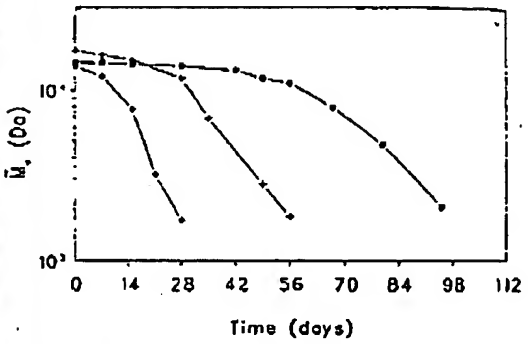


Fig. 1. Time-dependent $\bar{M}_w$ change of microspheres prepared with different types of polymers showing the effect of polymer composition: PLGA50:50-14 kDa (◇), PLGA75:25-17 kDa (+), PLA-14 kDa (●).

In addition to polymer composition, the initial $\bar{M}_w$ of the polymeric microspheres influenced substantially the total degradation time, although the overall degradation pattern followed a similar type of kinetics for the low and the high molecular weight particles, e.g. PLGA50:50-14 kDa versus PLGA50:50-35 kDa, PLGA75:25-17 kDa versus PLGA75:25-69 kDa, and PLA-14 kDa versus PLA-130 kDa (Fig. 2). As expected, PLA-130 kDa

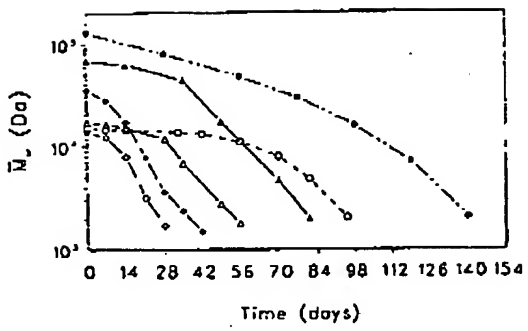


Fig. 2. Time-dependent $\bar{M}_w$ change of microspheres prepared with different types of polymers showing the effect of the initial molecular weight for various polymer compositions: PLGA50:50-14 kDa (◇), PLGA50:50-35 kDa (◇), PLGA75:25-17 kDa (△), PLGA75:25-69 kDa (△), PLA-14 kDa (○), PLA-130 kDa (●).

microspheres showed a very slow change in $\bar{M}_w$. Complete polymer erosion to water soluble oligomers was not finished up to day 140. Compared to the low molecular weight PLA-14 kDa, however, PLA-130 kDa hydrolysis was more pronounced during the initial part of the profile where no typical lag time could be detected. As a result of the sustained degradation, the polymeric microspheres studied were considered particularly useful for controlled antigen delivery over a period of several months. It was interesting to see that within the range of polymers studied an approx. 2-fold increase in $\bar{M}_w$ prolonged the time of degradation by 2 weeks (PLGA50:50-14 kDa versus PLGA50:50-35 kDa), at a 4-fold increase in $\bar{M}_w$ a prolongation of approx. 4 weeks (PLGA75:25-17 kDa versus PLGA75:25–69 kDa), and at a 9-fold increase in $\bar{M}_w$ a prolongation of degradation by approx. 6 weeks (PLA-14 kDa versus PLA-130 kDa) could be observed.

3.2. Morphology and size of the microspheres

It is reasonable to assume that surface morphology and, even more, microsphere size may play a crucial role for antigen delivery and presentation. Therefore, two different classes of microsphere sizes, one in the range of approximately 1–10 μm and the other of 10–100 μm, were prepared by the two preparation methods of spray-drying and coacervation, respectively (Fig. 3). Spray-drying produced smaller microspheres with the majority of particles below 5 μm, and coacervation yielded coarser microspheres, with the size distribution being a function of polymer $\bar{M}_w$ and of process parameters [34]. In general, the low $\bar{M}_w$ polymers gave a microsphere size in the range of 8 to 60 μm, whereas those of high $\bar{M}_w$ (PLGA75:25–69 kDa and PLA-130 kDa) ranged from 10 to 90 μm (Fig. 3). It is noteworthy that particle size distributions determined by laser light scattering and from SEM micrographs were in good agreement. Spray-dried microspheres exhibited a smooth and non-porous surface. In the case of coacervated microspheres, a similarly smooth surface morphology was detected when the antigen was microencapsulated as particulate material. On the opposite, when the antigen was entrapped as aqueous solution, a more porous surface was revealed on the SEM-micrographs.

Clearly, the use of the two different preparation techniques allowed microspheres with a wide range of polymer molecular weights and two distinct classes of particle sizes to be obtained. Both features are considered of prime importance for parenteral vaccine delivery systems.

3.3. Antigen microencapsulation and in vitro release

On the basis of the results obtained from polymer degradation and particle size distribution, the two antigens P30B2 and TT were microencapsulated using low molecular weight PLGA50:50-14 kDa and PLGA75:25-17 kDa, and a high molecular weight PLA-130 kDa. Both spray-drying (SD) and coacervation (CO) were applied. The various preparations are summarized in Table 1. For purely practical reasons, antigen powders rather than solutions were used in coacervation of PLGA75:25-17 kDa and PLA-130 kDa. It appears though, that the particulate antigens were less efficiently microencapsulated than the corresponding aqueous solutions. This difference is more significant with the synthetic antigen (27% for CO- PLGA75:25-17 kDa versus 57% for CO-PLGA50:50-14 kDa). It also appears that the efficiency to encapsulate the synthetic antigen is lower than for the natural high molecular weight TT.

Comparing the two encapsulation techniques, no

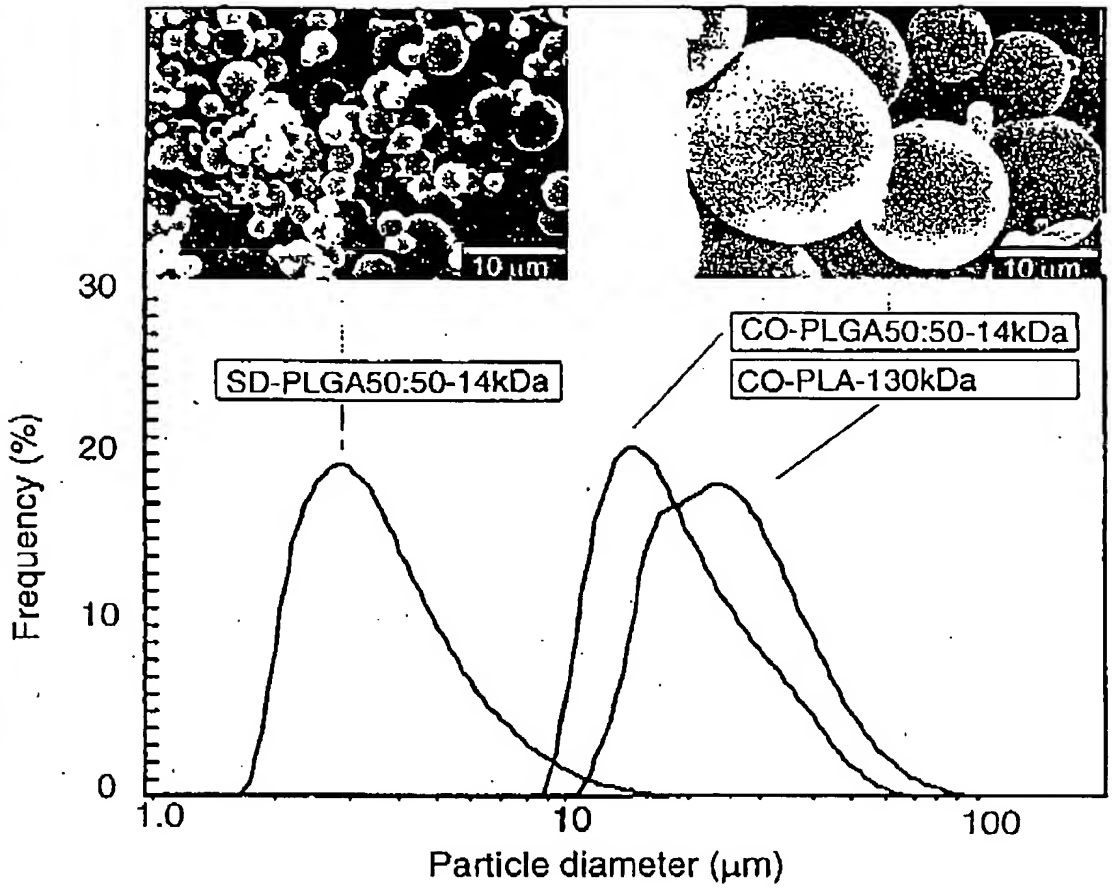


Fig. 3. SEM micrographs and particle size number distribution plots of typical microspheres prepared by spray-drying (SD-PLGA50:50-14 kDa) and by coacervation (CO-PLGA50:50-14 kDa, and CO-PLA-130 kDa).

significant difference in entrapment efficiency is observed if the antigens are processed in solution. Given the different preparation techniques, i.e. SD and CO, with the antigen for microencapsulation being in particulate form and in solution, an affect of the polymer composition on loading efficiency cannot be revealed unambiguously. Finally, with a few exceptions only, the loading efficiencies attained were satisfactory, and the actual loadings allowed to inject between 5 and 15 mg of P30B2-microspheres and between 1 and 4 mg of TT-microspheres per animal in the immunization studies.

Antigen release kinetics is considered one of the key parameters for vaccine delivery systems. Pulsatile release was actually achieved in the release of the 16 kDa P30B2 from the PLGA50:50-14 kDa microspheres (Fig. 4). After an initial burst release, which occurred during the first 1–2 days, a latency period of about 2 weeks was observed with very little antigen release. Release was completed after 7 weeks. The two release profiles obtained from the PLGA50:50-14 kDa microspheres prepared by spray-drying and by coacervation were very similar. For the coacervated microspheres, the onset of the second release pulse was slightly shifted to the right. Clearly, the term release pulse in understood in this work in a broad sense. The coacervated microspheres also gave rise to a substantially higher burst (20% of

Table 1
Antigen loading of microspheres

Antigen[a]	Polymer[a]	Microencapsulation technique[a]	Physical state of antigen for microencapsulation[b]	Loading (μg/mg microspheres)	Loading efficiency (%)[c]
P30B2	PLGA50:50	SD	Solution	3.51	54
	PLGA50:50	CO	Solution	6.00	57
	PLGA75:25	SD	Solution	6.59	60
	PLGA75:25	CO	Powder	2.06	27
	PLA	CO	Powder	2.64	26
TT	PLGA50:50	SD	Solution	18.20	90
	PLGA50:50	CO	Solution	8.50	85
	PLGA75:25	SD	Solution	21.50	100
	PLGA75:25	CO	Powder	7.00	70
	PLA	CO	Powder	5.80	58

[a]Abbreviations used are described in the experimental section; $\bar{M}_w$ of PLGA50:50 is 14 kDa, $\bar{M}_w$ of PLGA75:25 is 17 kDa and $\bar{M}_w$ of PLA is 130 kDa.
[b]The antigens were microencapsulated either as a 1–2% (w/v) aqueous solution or as a milled powder with particle size < 20 μm.
[c]The loading efficiency is defined as the percentage of the actual loading with respect to the theoretical loading.

the actual dose), than the spray-dried microspheres (10% of the actual dose). This difference may be explained by the slightly porous morphology of this particular MS-preparation (see under Section 3.2). It is also noteworthy that the total amount of P30B2 released from the coacervated PLGA50:50-14 kDa microspheres exceeded the measured loading by about 20%. This indicates that the recovery by the filtration method used to extract this antigen was maximal 80% with this particular preparation. The higher burst release and the underestimated loading of the coacervated PLGA50:50-14 kDa microspheres must have both contributed to the fact that this preparation exhibits an area under the second release pulse similar to the spray-dried PLGA50:50-14 kDa, although the actual loading of the coacervated microspheres exceeded that of the spray-dried product by about 40%.

While the microencapsulation method essentially affected the extent of the burst release, the polymer composition greatly determined the overall release kinetics, particularly the onset and duration of the second release pulse (Fig. 5). As mentioned before,

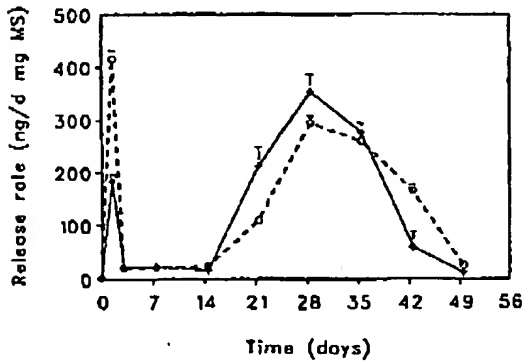


Fig. 4. In vitro release rates (in ng antigen per day and per mg microspheres) of P30 B2 from spray-dried (+) and coacervated (O) PLGA50:50-14 kDa microspheres. Error bars indicate standard deviations with $n = 3$.

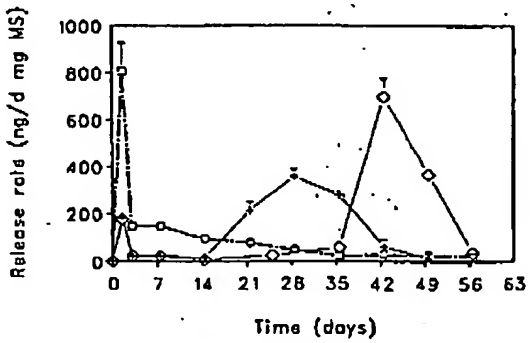


Fig. 5. In vitro release rates (in ng antigen per day and per mg microspheres) of P30B2 from spray-dried PLGA50:50-14 kDa (+) and PLGA75:25-17 kDa (◇), and from coacervated PLA-130 kDa (□) microspheres. Error bars indicate standard deviations with $n = 3$.

the onset and maximum of the second pulse for the PLGA50:50-14 kDa appear around day 14 and day 28, respectively, while for PLGA75:25-17 kDa, these two time points are shifted to day 35 and day 43. In contrast, for the slowly degrading PLA-130 kDa, no additional release pulse was detected within the time period studied. For both the PLGA50:50-14 kDa and PLGA75:25-17 kDa microspheres, the onset of the second pulse corresponded to a $\bar{M}_w$ of about 7000 to 8000 Da during the in vitro polymer degradation, while the maximum release rate was measured at a time point corresponding to a $\bar{M}_w$ of about 2000 to 4000 Da. From the polymer degradation profiles, the second release pulse from the PLA-130 kDa microspheres was expected after about 4 months, though the experiment was stopped before that time point.

Similarly to P30B2, TT release was not primarily affected by the preparation method and, hence, by the size of the microspheres, but by the type of polymer (Fig. 6). While the onset and duration of the second TT pulse from PLGA50:50-14 kDa microspheres was virtually identical to that observed for P30B2 with this same polymer, this similarity was not found for the PLGA75:25-17 kDa microspheres. For TT, the second release pulse from PLGA75:25-17 kDa microspheres started on day 56 and ended on day 84, corresponding to a shift of approximately 3 weeks with respect to P30B2. When comparing TT release profiles to microsphere degradation, a close correlation between the second release pulse and the final stage of polymer hydrolysis was observed. Clearly, the second TT pulse started when microsphere degradation reached a $\bar{M}_w$ of 2000 to 3000 Da. Although a $\bar{M}_w$ of 1000 Da was considered as complete polymer erosion, TT release was not yet finished at this time point but continued for an additional 4 weeks. For TT, the total amount released and the quantity determined as loading were in close agreement, indicating that the recovery of the extraction method used was in the order of 90–100% for this large protein.

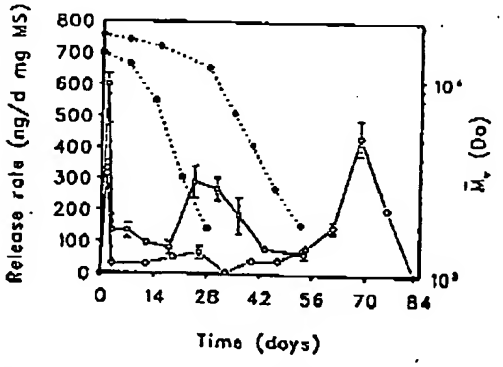


Fig. 6. In vitro release rates (in ng antigen per day and per mg microspheres) of TT from spray-dried PLGA50:50-14 kDa (○) and coacervated PLGA75:25-17 kDa (◇) microspheres, and change in $\bar{M}_w$ of the corresponding microspheres made of PLGA50:50-14 kDa (●) and of PLGA75:25-17 kDa (◇).

The excellent correlation between the second release pulse and the polymer degradation time for both antigens may indicate the feasibility to design PLA/PLGA-microspheres with well defined pulses at any desired time point, e.g. after 1, 2, 3, 6 months etc., corresponding to the degradation profiles in Fig. 2. However, although the onset and maximum of the second release pulse were well defined, the duration of the pulse extended over 2–4 weeks.

3.4. Immune response

Anti-TT and anti-P30B2 antibody responses were determined for all of the experimental groups at intervals of 2–4 weeks, up to week 45 for TT and week 28 for P30B2. In this contribution, the immunological response is expressed in terms of antibody titers.

For the synthetic and weakly immunogenic P30B2, the microsphere size did not appear to affect the general time course of antibody titers, although a slightly slower increase of the titers was observed with the larger size particles prepared by coacervation (Fig. 7). After 6 weeks, however, the two microsphere preparations showed very similar profiles. A comparison with the profile obtained with the IFA-formulation indicated a kinetic difference in the immune response. With IFA, the antibody level approached the maximum 4–6 weeks after immunization and started to decrease significantly after 4 months. With the microspheres, however, the antibody levels remained elevated over the entire time period studied (28 weeks).

The effect of the polymer type on anti-P30B2 specific antibody levels is presented in Fig. 8. Here again, significant differences between the profiles

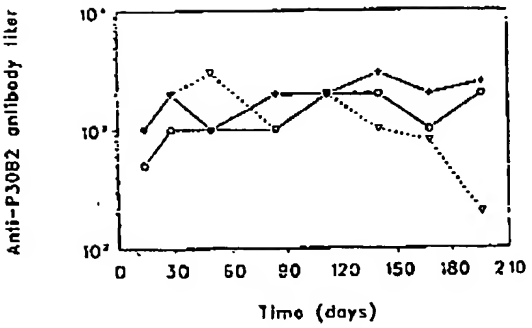


Fig. 7. Time course of anti-P30B2 antibody response in BALB/c mice upon injection of 50 μg P30B2 in different formulations showing the effect of the microencapsulation method and the related particle size: spray-dried (+) and coacervated (O) PLGA50:50-14 kDa microspheres, and reference formulation IFA (∇). Individual curves represent the geometric mean of antibody titers obtained from each group by ELISA.

were noticed in the early stage of the immune response, i.e. during the first 3 months. The fast degrading small-sized microspheres (SD-PLGA50:50-14 kDa) gave rise to an immediate and strong antibody response comparable to IFA. By contrast, the slower degrading PLGA75:25 microspheres (SD-PLGA75:25-17 kDa) and, even more, the larger size CO-PLA-130 kDa microspheres produced a more gradual increase in antibody titers with

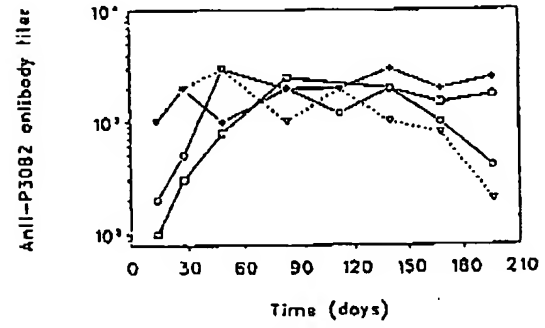


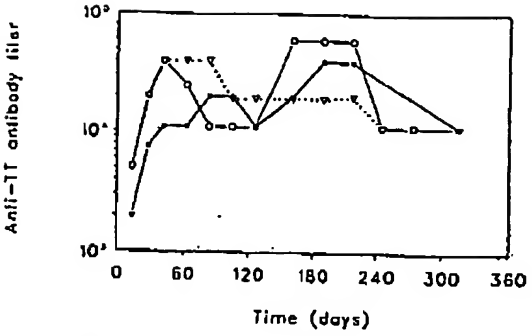
Fig. 8. Time course of anti-P30B2 antibody response in BALB/c mice upon injection of 50 μg P30B2 in different formulations showing the effect of the polymer type and the related release characteristics: spray-dried PLGA50:50-14 kDa (+), spray-dried PLGA75:25-17 kDa (O) and coacervated PLA-130 kDa (□) microspheres, and reference formulation IFA (∇). Individual curves represent the geometric mean of antibody titers obtained from each group by ELISA.

the maximum reached only 6 weeks (for SD PLGA75:25-17 kDa) or 12 weeks (for CO-PLA-13 kDa) after immunization. While for the PLGA50:5(14 kDa and the PLA-130 kDa microspheres th antibody levels remained elevated over the entir period studied, they tended to decrease after 1 weeks postimmunization in the case of PLGA75:25 17 kDa, similarly to those measured in the IFA group. Clearly, the expected release pulse from th PLA polymer after approximately 4 months eithe did not materialize in vivo or had no effect on th antibody titer. For the PLGA50:50-14 kDa an PLGA75:25-17 kDa microspheres, the determine release pulse was possibly too close to the initi burst release to exert an influence on the antibod titers. In mice, no booster effect could be observe with any of the microsphere preparations after singl injection, which contrasts the data of the in vitr experiments.

The data of the immune response unambiguousl demonstrated the immunostimulating properties c all the microsphere preparations. The observed di ferences between the various formulations wer essentially limited to the kinetics of the early stag antibody response. Larger and slowly degradin microspheres induced antibody titers more slowl than the small-sized and fast degrading micr spheres.

In contrast to the synthetic P30B2-formulation the TT microspheres generated more distinct kinet antibody profiles. Typically, the PLGA75:25-17 kD microspheres produced a vague anti-TT boost effect between days 120 and 180 (Fig. 9). In th initial stage of the profiles, a significantly fast antibody response was measured in the group of th small-sized microspheres (SD-PLGA75:25-17 kD On the other side, the maximum antibody titer an duration of the response did not appear to b influenced by the particle size (SD- PLGA75:25-1 kDa versus CO-PLGA75:25-17 kDa). The micr sphere formulations elicited antibody responses com parable to those obtained with the convention vaccine (TT-alum).

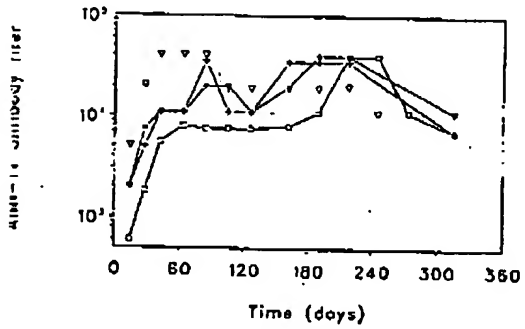
The influence of polymer composition on t antibody response is illustrated in Fig. 10, wi large-sized microspheres prepared by coacervati No difference was observed between the two typ of copolymers, the PLGA50:50 and PLGA75:25.



g. 9. Time course of anti-TT antibody response in BALB/c ice upon injection of 20 μg TT in different formulations owing the effect of the microencapsulation method and the lated particle size: spray-dried (○) and coacervated (●) LGA75:25-17 kDa microspheres, and reference formulation um (▽). Individual curves represent the geometric mean of ntibody titers obtained from each group by ELISA.

ry slight boosting effect was also noticeable with reparation PLGA50:50-14 kDa, as pointed out ready for the PLGA75:25 microspheres. With the owly degrading and releasing PLA microspheres, ntibody titers increased much slower and reached e maximum level only after about 220 days. Again, slight booster effect occurred between days 180 d 220.

Generally, all TT microsphere formulations eli-



. 10. Time course of anti-TT antibody response in BALB/c e upon injection of 20 μg TT in different formulations wing the effect of the polymer type and the related release racteristics: coacervated PLGA50:50-14 kDa (+), GA75:25-17 kDa (●) and PLA-130 kDa (□) microspheres, reference formulation alum (▽). Individual curves represent geometric mean of antibody titers obtained from each group ELISA.

cited a high antibody response comparable to the one obtained with the TT adsorbed on alum, and antibody levels remained elevated over a long period of time. Significant differences in antibody levels were observed only in the early stage of the immunological response, but not at later times. The differences in antibody titers in the initial phase can be explained by both the two classes of particle sizes used, and by the different in vitro antigen release kinetics in combination with polymer degradation. The small-sized microspheres gave generally faster antibody response than the larger microspheres (Fig. 9), and the slower initial TT-release from PLA-130 kDa as compared to PLGA50:50-14 kDa and PLGA75:25-17 kDa is reflected by a slower rise in antibody levels (Fig. 10).

4. Discussion

To rationalize the design of antigen delivery microspheres, this study aimed at a better understanding of the correlation between relevant microsphere characteristics, such as degradation time, particle size and release profile, on the one side, and the time course of the immune response, on the other side. Although an increasing number of studies on antigens microencapsulated into biodegradable microspheres have been published over the past 5 years, only very few have made attempts to investigate the influence of relevant microsphere characteristics on the immunological response [1,30,31].

The possibility of controlling antigen release through the degradation time of polymeric microspheres represents an attractive feature for antigen delivery. As polyester degradation mechanisms and time depend on processing conditions and sample dimensions, data obtained from native polymer powder or particular forms of implants are not necessarily representative for microsphere erosion [24]. Therefore, an in vitro degradation study was conducted with a series of PLA- and PLGA-based microspheres prepared by coacervation. The degradation profiles showed a uniform and consistent pattern revealing the importance of polymer hydrophobicity and molecular weight (Fig. 1 and Fig. 2). An increasing number of glycolide units renders the polymer more hydrophilic, resulting in a more

pronounced water uptake [21,27,38]. With increasing hydration, the glass transition temperature, T_g, decreases below the incubation temperature of 37°C, shifting the PLGA chains into a rubbery state and facilitating hydrolytic cleavage [18]. Polyester hydrolysis was reported to occur prior to polymer mass loss, whereby, in general, the change in $\bar{M}_w$ follows first-order kinetics [25]. Our data suggest that such an exponential decrease is not observed from the very beginning but follows a lag period of several days to several weeks with little change in $\bar{M}_w$ occurring. For the low and intermediate molecular weight microspheres, this initial phase primarily depends on the type and hydrophilicity of the polymer. A more pronounced change in $\bar{M}_w$ in the first few weeks was noticed though for the high molecular weight PLA. This finding is consistent with the reported observation of a faster degradation rate in high molecular weight polymers [21]. The different degradation kinetics between PLA-14 kDa and PLA-130 kDa might be explained by the substantial amount of residual solvents present in the PLA-130 kDa (3% DCM and 5% OMCTS), as compared to PLA-14 kDa microspheres (0.2% DCM and 1% OMCTS), contributing to a decrease of T_g and, hence, facilitating water uptake into the partially rubbery polymer bulk [34].

The accelerated degradation rate in the terminal stage of the profiles is in agreement with other reports and has been explained by the presence of increasing amounts of acidic degradation products or of Na^+ ions from the buffer [16,23,28,39]. More specifically, autocatalysis by acidic oligomers accumulated in the polymer bulk due to their reduced diffusion and dissolution characteristics at low pH has been suggested [25]. In our degradation experiments, the medium was regularly replaced by fresh buffer. Nevertheless, in the later stage of degradation, the buffer capacity was not sufficient to stabilize the pH around 7. In a recent study, unambiguous evidence was provided that the pH lowered by degradation products altered the degradation kinetics of microspheres [28]. This fact was illustrated by the large differences in the erosion profiles between particles kept either at constant pH in a dialysis bag or under non-constant pH conditions in buffered saline, becoming increasingly acidic through the accumulation of degradation products.

Knowledge of the polymer degradation tim enabled the selection of appropriate polymers f antigen microencapsulation, which were expected provide release pulses after 1, 2 and 4–6 mont The striking concurrence between the polymer degr dation time (Fig. 1 and Fig. 2) and the secon antigen release pulse of both antigens studied (Fig 4–6) is of prime importance for programming p tential booster doses from this delivery system. Th possible mechanisms responsible for the pulsati release behavior of peptides and proteins from PL/ PLGA microspheres have been discussed by seve authors [13,26,27,40]. The three distinct phases protein release observed have been described as t the initial burst release with the diffusion of acti compound located near the microsphere surfa during polymer hydration, (ii) the latency phase wi inhibited diffusion, and (iii) the release pulse due biodegradation and polymer mass erosion. It generally assumed that in the latent phase some ty of interactions between the negatively charged pol meric carboxyl groups and the protein must reta the active compound. Specifically, an ion exchan mechanism has been suggested [27]. If it is inde this mechanism which is mainly controlling t release of the actual antigens, the timely differen in the appearance of the second release pulse b tween TT and P30B2 might be explained by th molecular weight difference. The larger TT (1: kDa) must exhibit, at a given pH, a higher density ammonium groups able to interact with the pol meric carboxyl groups, as compared to the 16 kl P30B2. This would indeed result in a strong interaction with the larger TT. The formation of ionic complex between the carboxyl group carryi polymer and the protonated protein is, in gener expected within the approximate pH-range of 3. 5.5, close to or below the p*I* of the protein (e.g. p*I* TT = 5.1), and above the pK_a of the glycolic a lactic acids. In the case of charge separation on t protein, reaction with the anionic oligomers may al occur at higher pH. With respect to polymer degr dation, this may signify that proteins of a small size or of a lower p*I* will dissolve from the ion oligomer-protein complex at an earlier stage th larger proteins.

This interpretation of the latency phase obser in this study suggests a pH-microenvironment insi

ne polymer particles with a lower pH than that of he release medium. Such an acidic microenviron-ient has also been suggested by other authors 16,29]. The generation of high amounts of acidic legradation products inside the microspheres or in 'ie incubation medium has caused well founded oncerns about the stability of unreleased and re-:ased proteins [4,16,28,29]. With the analytical ethods used here, no degradation of the two ntigens could be detected. This does not mean, owever, that alteration of antigenicity or even mmunogenicity may be excluded during the release. hus, a much more complete set of analytical ethods would have to be used to detect degradation levant for the biological activity. Previous studies y our group and by others have focused on TT-ability in aqueous solutions and under freeze-dry-g conditions [4,41]. Presently, several groups are vestigating intensively TT stability within biode-adable microspheres. As TT is not likely to drolyze under the actual release conditions, co-lent intermolecular bonding or simple physical gregation between TT molecules or between TT d oligomers are also considered.

In addition to the pulsatile antigen release, the rticle size is generally expected to influence the mune response. It was shown that small-sized rticles, i.e. <10 μm, are very efficiently taken up macrophages resulting in improved antigen pre-ntation and enhanced adjuvant effect [42,43]. Our ta emphasize that the particle size, within the ge tested, exerts no effect on the maximum tibody titers nor on the duration of the response. e observed, however, that small microspheres, e.g.)-PLGA50:50-14 kDa, elicited 5- to 10-fold higher tibody titers at early time points than those de-ted with larger microspheres. Subsequently, anti-dy titers in the large-sized microsphere groups gan to rise so that no significant difference existed tween small and larger microspheres after several nths (Figs. 9 and 10), which is in agreement with er studies [43].

The ability of a single injection of microspheres to vide distinct and timely release pulses of mi-ncapsulated antigen may greatly facilitate future cine development. The antibody response ob-ed with different formulations during the time rval tested reflects, to some extent, the release properties of the microspheres. However, the release pulses measured in vitro induce only a vague booster effect in mice. It is not clear yet if this is a consequence of an inappropriate onset of the release pulses, the extended duration of the pulses, or the loss of immunogenicity of the antigens at later time points. To our knowledge, no unambiguous booster effect after single parenteral administration has been shown so far in similar type of investigations [2,30]. Considering all of the individual microsphere preparations, no essential differences were observed in the maximum antibody responses. In a previous study by our group, we have shown that the use of microsphere mixtures consisting of different types of polymeric microspheres produce substantially stronger immune responses than the individual microsphere types [36]. Besides the very high and sustained antibody titers, T-cell proliferative responses were greatly enhanced in the groups of mice receiving a single injection of a microsphere mixture. Clearly, 45 weeks after immunization, the proliferative response of the microsphere mixture group and the group having received three injections of alum adsorbed TT (at weeks 0, 5 and 16) was comparable. Although the microsphere mixtures elicited greatly enhanced and more prolonged immune responses, there was no indication of a booster effect. This might indicate that the overlapping of the release pulses from the different individual microspheres types may not only enhance the maximum response, but may hide simultaneously any booster effects.

5. Conclusions

With the two antigens studied, the natural tetanus toxoid (TT) and a weakly immunogenic malaria peptide (P30B2), a clear correlation between the in vitro polymer degradation and the in vitro antigen release profile was found. Antigen release follows a pulsatile pattern with a first pulse representing the so-called burst effect and a second pulse determined by the polymer degradation time. The antigen containing microspheres exerted an important immunostimulating effect in terms of antibody response. Antibody titers remained elevated over the entire time period studied, i.e. up to 45 weeks, and were comparable to the P30B2 in IFA and TT on alum.

However, the pulsatile in vitro release profile generated only a slight boosting effect in vivo. Nonetheless, the time course in antibody titers could be explained, at least partly, by the particle size and the release behaviour. A slower increase in antibody titers was observed with the microspheres of larger size or exhibiting a slower release. Immediate and highest antibody levels were elicited with the small-sized fast degrading microspheres.

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